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RELATIONSHIP BETWEEN THE REPRODUCTIVE AXIS AND THE ADRENAL AXIS OF THE GILT: ONTOGENY OF FUNCTION AND EFFECT OF ACTH ACTIVATION

BY

FRANKLIN DAVID EVANS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

ANIMAL PHYSIOLOGY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

SPRING 1987

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled RELATIONSHIP BETWEEN THE REPRODUCTIVE AXIS AND THE ADRENAL AXIS OF THE GILT: ONTOGENY OF FUNCTION AND EFFECT OF ACTH ACTIVATION submitted by FRANKLIN DAVID EVANS in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL PHYSIOLOGY.

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ABSTRACT

The objective of this research was to characterize development of the adrenal axis with respect to development of the reproductive axis and to determine if activation of the adrenal axis affects the function of the reproductive axis of the gilt.

In the first study, 17β-estradiol receptor concentrations in the cytosolic fraction of regions of the hypothalamus and pituitary were determined at 4 wk intervals from 4 to 28 wk of age. No significant changes ($P>0.05$) in receptor concentrations with age were detected.

In the second study development of the circadian rhythm of cortisol was monitored from 4 to 28 wk of age. The profile of cortisol progressed from non-rhythmic at 4 wk to adult rhythmic profiles at 28 wk consisting of a morning peak, small afternoon peak and evening trough. Non-cortisol binding globulin bound fractions of cortisol closely mirrored total cortisol profiles.

In the third study 56 gilts were assigned to four treatments consisting of one injection of adrenocorticotropic hormone (ACTH), ten injections of ACTH or their respective saline vehicle controls. There were no effects ($P\geq0.05$) of ACTH on basal or on estradiol stimulated luteinizing hormone plasma concentrations. Injection of 40 IU ACTH had no effect on subsequent plasma

concentrations of unstimulated cortisol.

In the fourth study, growth curves for the heart, lungs, spleen, liver, kidneys, thyroid, pineal, adrenal glands and reproductive tract including the components, uterus, fallopian tubes and ovaries were determined in gilts from 4 to 28 wk of age. Relative growth curves for the reproductive tract components were biphasic and increased while for all other organs relative growth was constant or decreased with age. Histological examination of the adrenal cortex, including zonal thickness and nuclear to cell volume ratios, failed to show increases that might be associated with growth spurts observed in the reproductive tract.

There was no evidence from these studies to suggest that any component of the adrenal axis distal to ACTH release has an effect on reproductive development in the gilt.

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I. INTRODUCTION

THE NEED FOR EARLY PUBERTY

One area of concern that continues to occupy researchers involved in swine production is that of reproductive inefficiency. Increasing female reproductive efficiency is probably the most effective way of increasing the profitability of a swine herd (English et al. 1978). Contributing to reproductive inefficiency in a swine herd is the high replacement rate of sows with young gilts. The annual sow replacement rate is reported to average 40%, but varies greatly from herd to herd even in countries advanced in swine production such as Denmark, United States and Canada (Nielsen 1980; Christenson 1986).

In modern swine production systems, hogs reach market weight between 90 and 100 kg livew eight at an age of approximately 150 d. If replacement gilts are to be selected from the pool of market hogs, few if any gilts will have reached first estrus by this time (English et al. 1978). Under feed and management systems commonly employed in Europe and North America, first estrus is reported to occur at an average of approximately 200 d of age (Dyck 1971; Anderson and Melampy 1972). Under commercial swine production systems larger numbers of gilts than are actually required for sow replacement are withheld from market to ensure that the required number of replacements are obtained. If traditional methods of swine

production are followed, the recommendation is to wait until second or third estrus before selection and mating occurs (Jamieson 1978; English et al. 1978). Any gilts not selected for breeding at this time would be sold as market hogs. Financial losses to the producer result from housing and feeding these surplus gilts and also because of the loss in carcass quality when these heavy hogs, not selected as breeding stock, are returned to the market chain. Clearly, a more efficient system of obtaining herd replacements would be beneficial.

The recommendation to delay breeding gilts until the third estrus is based on the assumption that ovulation rates increase with each subsequent estrus up to the third or fourth estrus after puberty (Robertson et al. 1951; Zimmerman et al. 1957; Haines et al. 1959). However, Dyck (1971) suggested that ovulation rate remained constant over the first three estrus cycles. The method used to determine ovulation rate by many researchers involved slaughter of the gilts at the third or fourth estrus. Ovulation rate at the time of slaughter was estimated by counting the number of corpora lutea while ovulation rate for previous estrus periods was estimated by counting corpora albicantia. Dyck (1971) suggested that some of the corpora albicantia were not detected, accounting for the estimated lower ovulation rate for previous estrus periods. In spite of this controversy, it has been estimated that economic advantage may still remain with

the earliest possible mating (MacPherson et al. 1977).

The ideal swine production system would be one in which gilts cycle at least once by the time market weight is reached. Final selection for herd replacements could then take place at this time, from a potentially larger pool of animals and from gilts that are known to have cycled. Under such a system it may even be advantageous to breed at first estrus, selecting gilts for replacement from those that conceive, and returning the others to the market chain before carcass quality has decreased. In order to accomplish this goal however, those factors that may reduce age at first estrus must be identified and their effects optimized.

FACTORS AFFECTING AGE AT ONSET OF PUBERTY

Confinement

Confinement housing, accompanied by intensive forms of animal production generally improve overall management and efficiency of swine production systems. However, confinement has been reported to be detrimental to sexual development in gilts (Christenson 1981). Christenson (1981) observed that the percentage of gilts with regular estrous cycles from 2 to 9 m of age was consistently lower for confinement-reared gilts compared to nonconfinement-reared gilts.

There is evidence to suggest that social factors may be involved in the effects of confinement on reproduction.

Ford and Teague (1978) reported that when floor space was restricted to one-half the space allowed to control gilts, average age at first estrus and number of gilts reaching estrus was not different. The number of gilts housed per pen however has been found to affect reproductive performance. Christenson (1984) observed that the number of gilts that were cyclic at 9 m of age was higher when housed in groups of 9, 17 or 27 as compared to groups of 3 (78, 80 or 81% compared to 57% respectively). On the other hand Cronin et al. (1983) reported that when gilts were housed in groups of greater than 50, reproductive performance was depressed. Christenson and Ford (1980) have suggested that the increased levels of stress associated with some aspects of confinement housing may increase adrenocorticosteroid production which could be responsible for delaying onset of puberty and reducing expression of estrus.

Season

Wiggins et al. (1950) were the first to report the effects of season on swine production. More recently, Scalon and Krishnamurthy (1974) studing the extent of reproductive tract development in gilts at market weight, reported that gilts slaughtered in the summer showed less development than those slaughtered during winter months. Similarly, Christenson (1981) reported that the incidence of normal estrous cycles at 9 m of age was less for gilts

reaching maturity during the summer than during the winter. In addition, this effect was observed under both confinement and nonconfinement conditions of housing. However, Tomes and Neilsen (1979) have observed that the delay in age at puberty was more pronounced under warm humid conditions than under cool dry ones, thus implicating temperature or possibly humidity as the determining factor rather than season. Even phase of the moon has been reported to influence age at first estrus and conception rate in dairy heifers (Roy et al. 1980). It is apparent that many environmental factors may have an effect on the reproductive process, either singly or in concert.

Light

Long photoperiods have been reported to increase the incidence of behavioral estrus, decrease age at puberty, increase ovulation rate and increase litter size (Klotchkov et al. 1971). With regard to reducing age at first estrus, the effects of long photoperiods have evoked much controversy. Hacker et al. (1974), Ntunde et al. (1979) and Diekman and Hoagland (1982) have reported that long photoperiods reduce age at first estrus whereas others have reported no effect (Kelly et al. 1982) or negative effects (Dufour and Bernard 1968).

The lack of agreement in the literature makes it difficult to draw conclusions concerning the effects of

photoperiod on onset of puberty. However, the inconsistent effects reported in the literature point to the possibility that parameters of light other than photoperiod may be important. There is increasing evidence that the major effects of environmental lighting may be to provide a "zeitgeber" or time giver to cue rhythmic processes involved in hormone release (Menaker et al. 1978). All of the above researchers in studying the effects of light on reproduction in the gilt have neglected the cueing function of light in entraining physiological processes. Researchers investigating the complex effects of environmental factors including light, temperature, season, social cues and feeding are required to take into consideration this aspect of control by environmental rhythmicities since each of these has been shown to be a potent zeitgeber to various physiological processes (Bunning 1973).

Nutrition

The results of experiments of the effects of feeding regime on age at puberty have been variable. Only a severe reduction in feed intake (about 50% ad libitum) significantly increased age at puberty (Haines et al. 1959). With intermediate levels of feed restriction (about 75% ad libitum) inconclusive results have been obtained. In some cases age at puberty was increased (Zimmerman et al. 1960; O'Bannon et al. 1966) while in others it was

decreased (Self et al. 1955; Aherne et al. 1976) or was not changed (Sorenson et al. 1961; Friend 1977). In a review of the literature Anderson and Melampy (1972) reported that restricting energy intake delayed puberty by an average of 16 d in nine experiments while in five other studies puberty occurred an average of 11 d earlier. Comparisons among these studies were difficult however, since there were many confounding variables including the level of restriction, age at which the restriction was applied and whether energy intake or total feed intake was restricted. In an effort to minimize the influence of these variables, den Hartog and van Kempen (1980) applied statistical adjustments to selected studies. The results of their review indicated that restricted gilts reached puberty 9 d younger and 19 kg lighter than full-fed gilts. Kirkwood and Aherne (1985) in a review of the effects of nutrition on puberty in the gilt have suggested that under normal conditions of feeding and management, nutrition will have minimal effects on reproductive development.

The effects of nutrition on the onset of puberty can be factored into the effects of the different characteristics of the diet such as energy density of the ration, protein content, protein to energy ratio and even amino acid composition. Holness (1972) has implicated deficiencies of individual amino acids in the delay in the onset of puberty. Cunningham et al. (1974) reported that an increase in crude protein from 10 to 14% decreased age

at first estrus by 18.9 d whereas O'Bannon et al. (1966) suggested that a high energy intake was associated with increased growth rate and earlier onset of puberty. Increased feed intake may also be accompanied by an increase in mixed function oxidase activity which results in increased steroid metabolism by the liver (Dziuk 1982). In addition, changing adipose tissue stores, under conditions of increased fat turnover or mobilization could release stores of steroids to the plasma (Hillbrand and Elsaesser 1983) and increased levels of body fat have been associated with increased aromatization of steroids (e.g. androgens to estrogens) in adipose tissue (Nimrod and Ryan 1975). The final result could be a temporary or perhaps prolonged change in steroid milieu with a corresponding alteration in reproductive function.

Boar Exposure

The first recognized and documented effects of the mere presence of the male animals changing the reproductive status of female animals was reported for mice and termed the "Whitten effect" (Whitten 1957). The Whitten effect is reported to be due to the production of unidentified non-steroidal, androgen-dependent pheromones in the urine of males that accelerate sexual development in juvenile females, induce estrus in anestrous females and alter duration of estrous cycles in cycling females (Bronson and Whitten 1970). In swine, a similar effect of

the boar on the induction of puberty in gilts has been noted. In swine, it has been reported that exposure of prepubertal gilts to mature boars induced an early onset of puberty (Brooks and Cole 1970; Bourn et al. 1975; Thompson and Savage 1978; Kirkwood and Hughes 1979).

It has been suggested that pheromones, responsible for chemical communication among many other species (Wilson and Bossert 1963), were responsible for the boar effect in the reduction in age at first estrus in gilts (Brooks and Cole 1970; Booth 1975). However there has not been definite identification of the chemical compound responsible for this effect in swine. The compound 5-alpha-androst-16-ene-3-one, the principle compound found in boar urine responsible for inducing standing behavior in female pigs and boar odour in pork meat (Melrose et al. 1971) was found not to be the factor responsible for early induction of puberty (Kirkwood and Hughes 1980). The compound 5-alpha-androst-16-ene-3-one may be merely a chemical "releaser" responsible for immediate behavioral responses (Wilson and Bossert 1963). Many other steroid-based potential pheromones have been identified chemically but few have been investigated as to their role in puberty induction (Thompson et al. 1972). It is also possible that sources other than the urine, such as saliva from the submaxillary glands may be the source of the pheromones that exert effects on the endocrine and reproductive systems responsible for onset of puberty

(Patterson 1968).

Transport and Stress

One effect on gilts that is confounded with boar exposure, apart from the as yet unidentified pheromones, is that of the stress involved in mixing gilts with mature boars. Bourn et al. (1974) reported that the transportation or herding of gilts from one area to another was at least partially responsible for the earlier onset of puberty. It was also concluded by Thibault et al. (1966), Signoret (1972) and Zimmerman et al. (1974) that the movement or mixing of animals does in itself have an effect on early induction of puberty quite apart from that involved with exposure to the boar. Certainly, with mice it has been shown that stress by restraint can re-establish normal estrous cycles in otherwise non cyclic females, increase litter sizes and increase the incidence of conception in cycling females (Paris and Ramaley 1974). Therefore, a variety of experimental circumstances have implicated stress as a factor which influences onset of puberty.

STRESS AND THE ADRENAL CORTEX

One of the predominant effects of applying a stressor to an animal is that it activates the adrenal cortex. Stressors that are not traumatic such as immobilization or exposure to cold are as effective at activating the

adrenal cortex as life threatening situations such as hemorrhage or exposure to potent toxins (Suzuki 1983). Even psychological stressors such as exposure of pigs to a novel environment are reported to be as effective as physical stressors in activating release of hormones from the adrenal cortex (Dantzer and Mormede 1985). Thus, activation of the adrenal gland during transport stress and perhaps boar exposure may play a role in the early onset of puberty in gilts.

THE ADRENAL CORTEX AND REPRODUCTION

The influence of the adrenal cortex on the function of the hypothalamo/pituitary/gonadal axis is still poorly understood. Activation of the adrenal gland by adrenocorticotropic hormone (ACTH), which is itself released from the anterior pituitary gland by corticotropin releasing factor (CRF), results in the release of steroid hormones from the inner two zones of the adrenal cortex, the zona fasciculata and the zona reticularis (Sayers and Portanova 1975). The outer zone, the zona glomerulosa secretes mineralocorticoid hormones and is largely independent of pituitary control (Long 1975). Production of the two other classes of adrenal hormones, glucocorticoids and sex steroids, is confined mainly to the reticularis and fasciculata and it is generally considered that both classes of these steroids can be produced by each of these layers (Vinson and Kenyon

1978).

Steroid production by the adrenal cortex is indeed varied and in mammals includes various estrogens such as estrone and estradiol-17β, androgens such as dehydroepiandrosterone, testosterone and androstenedione and glucocorticoids such as cortisol and corticosterone (Long 1975). The exact ratio of steroids produced is species dependent. For example, in the rat the predominant glucocorticoid is corticosterone whereas in man and the domestic pig cortisol is predominant (Vinson and Kenyon 1978). Hacker et al. (1973) reported that 12 wk old pigs housed at 20°C had serum concentrations of cortisol three to four times as high as concentrations of corticosterone (2.9 vs 0.9 μg dL^{-1}). Information regarding sex steroid production by the adrenal glands of the pig is lacking.

There are many reports in the literature which suggest that not only may the adrenals affect the reproductive axis by means of steroids unique to the adrenals but also through production of sex steroids. For example, Cutler and Loriaux (1980) have calculated that the adrenal cortex produces 10 μg of estrone per day in addition to the 25 μg of estrone that arises by peripheral conversion of adrenal androstenedione. Administration of large amounts of aromatizable adrenal androgens (Kraulis et al. 1978a) or deoxycorticosterone, an adrenal glucocorticoid (Kraulis et al. 1978b) have been reported to advance the

onset of puberty in female rats and to invoke an LH surge in estrogen-primed animals. Thus it seems that adrenal hormones directly, or their metabolites formed in a peripheral tissue, such as adipose tissue, may play a role in maturation of the hypothalamo/pituitary/ovarian axis leading to early onset of puberty.

Further evidence for the role of the adrenal glands in the onset of puberty has resulted from studies conducted with the rat (Gorski and Lawton 1973). These authors observed that puberty was significantly delayed by adrenalectomy at 18 or 25 d of age but not at 35 d of age. Adrenal autotransplantation at 18 d of age prevented the delay in puberty but was ineffective in the 25 d old group. They suggested that in the rat there is a period between 25 and 35 d of age during which adrenal secretions play a major role in the maturation of the hypothalamo/pituitary/ovarian axis. Also, since adrenal autotransplantation inhibited the adrenalectomy-induced delay in puberty onset and adrenal demedullation had no apparent effect upon puberty, the adrenal factor responsible for these actions appears to be of cortical origin rather than a catecholamine from the medulla.

THE ADRENAL AXIS AND THE REPRODUCTIVE AXIS

The hypothalamo/pituitary/adrenal axis undergoes progressive maturation during growth of an animal in a manner similar to the hypothalamo/pituitary/gonadal axis.

Both axes involve participation of the higher nerve centres of the brain, which influence synthesis and release of neurohormonal releasing factors, corticotropin releasing factor (CRF) for the adrenal axis and gonadotropin releasing hormone (GnRH) for the reproductive axis (Fink and Phil 1979; Sadow 1979). Releasing factors act upon different cell types in the anterior pituitary to release adrenocorticotropic hormone (ACTH) (adrenal axis) or the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (reproductive axis). These pituitary hormones in turn act upon either the adrenal glands or gonads to induce steroid hormone synthesis and release. These steroid hormones feed back, through both long loops and short loops, to moderate hormone release at several sites in the axis (Ramirez 1973; Sayers and Portanova 1975).

The development of the adrenal axis can be monitored by following the emergence of the rhythmic release of the major glucocorticoid. In the rat the corticosterone rhythm has been shown to gradually develop a distinct diurnal rhythm showing statistically significant diurnal variation at approximately 21 d of age and maturing into adult profiles shortly before or at the time of puberty (vaginal opening) at 35 d of age (Takahashi et al. 1979). In the human, maturation of the adrenal axis is accompanied by the development and the growth of the zones of the adrenal cortex, in particular, the zona reticularis (Dhom 1973).

In contrast to the progressive development of the adrenal axis the reproductive axis appears to develop in distinct spurts. Estrogen concentrations in the female rat are reported to rapidly increase from birth, reaching a peak at 21 d of age, decrease to baseline and then increase again at vaginal opening, at 35 d of age (Ramaley 1979). Similar profiles have been reported for the human (Ojeda et al. 1980) and for the gilt (Diekman et al. 1983).

According to the gonadostat theory (Ojeda et al. 1980) the first increase in plasma estrogen concentration is a result of increased release of LH and FSH since at this early age the estradiol negative feedback is relatively weak. Shortly thereafter, as the negative feedback effect matures and becomes maximally effective, basal gonadotropin levels drop precipitously followed by a decrease in serum estrogen concentrations. From this point until puberty, there is a progressive decrease in the negative feedback effect of estradiol on gonadotropin release. This allows serum gonadotropin levels to gradually increase and ovarian development progresses to the point where the positive feedback effect of estradiol triggers an LH surge and ovulation occurs. At this point behavioral estrus is displayed and puberty is said to have been achieved.

In addition to the gonadostat theory of sexual maturation other hypotheses have been suggested in an

attempt to account for other physiological events leading to the final maturation of the reproductive axis. These theories, as reviewed by Ramaley (1979), include the neuron theory, receptor theory, neuron modulation theories, peripheral interconversion of steroids theory, catechol estrogen theory, critical body weight or composition theory, and the involvement of the adrenal axis. More recently the circadian pacemaker theory has been suggested by Lehrer (1985).

Most of these theories have been based on studies centered on sexual maturation of the rat or human female. In swine credibility might be given to the role of the adrenal axis in determining the maturation of the reproductive axis since it has been repeatedly demonstrated that stress, possibly through the activation of the adrenal axis, is effective in inducing early puberty.

To date there has been little research into the effects of the adrenal axis on maturation or function of the hypothalamo/pituitary/ovarian axis during puberty in gilts. Although much reseach has been conducted into the relationship of these two axes in the rat and human, results have been inconclusive (Ojeda et al. 1980). Therefore, one objective of this study, was to map the development of the adrenal axis with respect to the development of the reproductive axis in maturing female swine. The second objective, was to determine if

activation of the adrenal axis by ACTH could alter the pattern and magnitude of LH release by the reproductive axis.

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II. ESTROGEN RECEPTOR CONTENT OF REGIONS OF THE HYPOTHALAMUS AND PITUITARY OF THE GILT FROM FOUR TO TWENTY-EIGHT WEEKS OF AGE

INTRODUCTION

Many theories exist to explain the central mechanism responsible for the onset of puberty (Ramaley 1979; Ojeda et al. 1980). Kato (1980) has suggested that the lack of steroid receptors in the juvenile mammal prevents feedback in the areas of the brain responsible for the regulation of reproductive function. It has been shown that the hypothalamic and pituitary content of 17ß-estradiol receptors (E_2R) increases with age in the developing female rat (Kato et al. 1974). This increase in E_2R content of regions of the hypothalamus regulating the release of GnRH would result in an increase in response to estradiol, ultimately leading to increases in gonadotropin release, follicular development and first estrus.

Within the hypothalamus of the adult female rat significant concentrations of E_2R have been found in the preoptic region of the anterior hypothalamus and the median eminence of the central hypothalamus (Vertes et al. 1973). These are also the respective sites at which the positive and negative feedback effects of estradiol on GnRH release are thought to occur (Ramirez 1973). The anterior pituitary, another site of gonadotropin control,

also contains concentrations of E_2R that increase prior to sexual maturity (McEwen 1976).

Unfortunately, most theories concerned with puberty onset and the role of E_2R in the various locations of the brain have been based on data collected from rats. The objective of this study was to determine whether changes in E_2R content occur in the anterior, central and posterior regions of the hypothalamus and in the anterior and posterior pituitary in gilts, from 4 wk to 28 wk of age.

MATERIALS AND METHODS

Thirty-five Yorkshire x Landrace gilts were randomly selected at weaning. Housing consisted of straw littered concrete pens in an enclosed, ventilated building maintained at 20°C throughout the trial which was conducted from August to January. A starter diet containing 18% crude protein (12.7 MJ DE kg^{-1}) was fed from weaning at 4 wk until 20 kg live weight. From 20 kg to 50 kg live weight a grower diet containing 16% crude protein (13.2 MJ DE kg^{-1}) was provided and from 50 kg to the end of the trial animals were fed a 14% crude protein (13.3 MJ DE kg^{-1}) finisher diet. The composition as well as analysis of these wheat, barley and soybean based diets are listed in the Appendix (Table 1, Table 2 and Table 3). Throughout the trial both feed and water were available ad

libitum. Feed intake, determined on a pen basis, as well as live weight was recorded weekly throughout the study.

Every 4 wk from 4 to 28 wk of age a group of 5 animals was slaughtered. After stunning with a captive bolt gun, the carotid arteries were severed and the head removed. The skull was opened and the ventral brain exposed. The pituitary gland, lying in the sella turcica was identified and removed and the anterior pituitary (AP) was separated from the posterior pituitary (PP). The hypothalamus was removed and separated into 3 blocks termed the anterior hypothalamus (AH), central hypothalamus (CH) and posterior hypothalamus (PH). In this study the AH contained the preoptic area and optic chiasm as well as the anterior hypothalamic area. The CH contained the median eminence while the PH contained the mammillary body. Thickness of the blocks ranged from approximately 4 mm in the 4 wk old animals to 8 mm in the 28 wk old animals. Tissue blocks weighed from 0.2 g to 0.5 g. Immediately after isolation, all tissues were placed in small plastic vials, snap frozen and stored in liquid nitrogen until assayed for receptor content.

The cytosol was prepared in the following manner with all operations carried out at 0-4°C. Tissue samples were placed frozen, directly into 4 mL of homogenization buffer (10 mM Tris-HCl, 1 mM EDTA, 3 mM sodium azide, 10 mM sodium molybdate, 10% v/v glycerol, pH 7.4 at 22°C, 3 mM dithiothreitol added just before use).

Homogenization took place in a glass-glass hand homogenizer (30 strokes) on ice followed by two rinses of 1 mL each of buffer. The homogenate was centrifuged at 1000 x g for 10 min and the resulting supernatant was centrifuged at 100,000 x g for 60 min to yield the supernatant cytosol fraction.

For the binding assay 200 µL of cytosol was added to 1.5 mL plastic tubes (Eppendorf). Tritiated estradiol was then added to these tubes in a 50 µL volume of homogenization buffer such that final incubation concentrations ranged from 0.05 to 5.0 nM. The [2,4,6,7,16,17-^{3}H]estradiol-17ß (140 C/mmole, Amersham), initially supplied at a purity of greater than 98%, was used as the tracer steroid without further purification. To determine the amount of non-specific binding another identical set of tubes was prepared but in which an additional 100 fold excess of unlabelled diethystilbestrol was added. The tubes were then vortexed, incubated at 37°C for 30 min to exchange steroids, rapidly cooled to 4°C and incubated overnight. To separate free from bound steroid 500 µL of 0.1% dextran (Dextran 500, Sigma, St. Louis, MO, USA), 0.1% BSA (Sigma) and 1.0% charcoal (Neutralized, Sigma) in 4°C homogenizing buffer were added to each tube containing cytosol. After vortexing, tubes were incubated for 10 min at 4°C and centrifuged at 12,800 x g (Eppendorf 5412 microcentrifuge) at 4°C. A 500 µL aliquot of the

supernatant was placed in a 6 mL scintillation vial and counted in a liquid scintillation counting system.

To serve as a receptor positive control, pig uterus was lyophilised, pulverised and stored as a powder at 0-4°C. Determinations conducted on this material were used to determine coefficient of variation between assays. This procedure has been described previously and the material was shown to be stable for periods of time in excess of 6 months (Koenders et al. 1981). The protein content of the cytosol samples was determined by the method of Lowry et al. (1951). Bovine serum albumin (BSA, fraction V, Sigma) was used as standard.

For receptor quantification and affinity determination the data was analysed by the method of Scatchard as described by Braunsberg and Hammond (1981). Specific binding of estradiol-17ß to unoccupied receptor sites was expressed in fmol mg^{-1} of cytosol protein. The results of the binding assays and Scatchard analysis of receptor concentration were evaluated by two way analysis of variance with repeated measure and differences evaluated by Student-Newman-Keuls' test (Steel and Torrie 1980). Since only one determination could be conducted for the pooled PP tissue samples these results were not included in the statistical analysis.

RESULTS

The relationship between number of receptors detected per tube and concentration of protein in lyophilized uterus used as control was found to be linear over the concentration range normally encountered in the assay (Fig. II.1). These determinations resulted in a coefficient of variation between assays of 9.9%.

Saturation curves for tissue samples from the AH, CH, PH, AP and PP are shown in Fig. II.2. From these curves it was observed that the range in concentrations of tritiated estradiol used for these binding assays was appropriate and fully saturating at the highest concentration. Scatchard plots indicating the affinity constant (Ka) obtained for representative samples of the same tissues are shown in Fig. II.3. Concentrations of estradiol receptors in the indicated regions of the hypothalamus and pituitary from 4 wk to 28 wk of age as well as pooled Ka values are presented in Table II.1. There was no significant change ($P=0.11$) in receptor content with age, however AP E_2R concentrations were significantly greater ($P<0.001$) than those of the tissues AH, CH or PH (10.41 $\pm$ 1.41 vs 6.48 $\pm$ 0.89, 6.74 $\pm$ 0.67 or 6.17 $\pm$ 0.58 fmol mg^{-1} protein).

Age at first estrus for gilts housed under the same conditions as animals in this study was 205.8 $\pm$ 3.4 d (mean $\pm$ SEM) (Chapter V).

DISCUSSION

It has been shown in the rat, that the anterior pituitary and ovary are capable of function far in advance of the age at which sexual maturity occurs. Included in this maturation is the ability of the ovary to respond to gonadotropin (Rennels 1951) and the ability of GnRH to release LH and FSH from the anterior pituitary (Odell and Swerdloff 1976). This early maturity extends to the GnRH producing neurons in the hypothalamus. It has been shown that GnRH producing neurons contain large concentrations of GnRH (Araki et al. 1975) as well as being in close proximity to a functional portal system (Florsheim and Rudko 1968) well in advance of vaginal opening.

From these and other findings it is quite clear that maturation of all levels of the reproductive axis from the gonads up to but not including the release of hypothalamic stores of GnRH occurs well in advance of first estrus. However, these stores of releasing hormone can be released by administering exogenous estrogen or electrically stimulating specific areas of the brain (Barraclough 1973).

This same concept of functionality of all components of the reproductive axis distal to GnRH release also applies to the gilt as well as the rat. In the gilt, the anterior pituitary is clearly able to produce gonadotropins as early as 3 wk of age (Chapter V). Also,

administration of GnRH can induce ovulation (Schilling and Cerne 1972) and administration of estrogen to gilts of at least 16 wk can induce estrus and ovulation. (Hughes and Cole 1978).

Implicated in the effects of estradiol on GnRH release are E_2R. The role and action of E_2R in the induction of cellular responses has recently been reviewed and revised (Walters 1985). Since it has been established that the quantity of receptors occupied determines the response of a particular tissue to a hormone it would be expected that an increase in receptor content would be associated with an increase in responsiveness of the tissue (Mulvihill and Palmiter 1977). In the rat it has been established that a gradual increase in hypothalamic E_2R content of the cytosol fraction occurs, reaching adult levels shortly before vaginal opening (Kato et al. 1974). It has been suggested that puberty is delayed for some time after apparent maturation of the pituitary and gonads due to the lack of E_2R in those regions of the hypothalamus responsible for the effects of estrogen on gonadotropin release (Ramaley 1979).

From the present study it appears that there is no progressive increase in receptor concentration content in any region of the hypothalamus or pituitary during sexual maturation of the gilt. If the gilt was similar to the rat, it would be expected that the preoptic area, found in the AH, and responsible for the positive feedback effect

of estradiol on GnRH release (Goodman 1978) would have a gradual increase in receptor content. Instead, relatively constant E_2R concentrations were found in the gilt except for minor fluctuations (Table II.1). These constant levels of E_2R were also found in the CH, the region in which the negative feedback effect of estrogen on GnRH release in the rat is reported to be located and in the PH, a region not thought to be a site of gonadotropin control (Ramirez 1973).

Similarly, no progressive increase in E_2R was detected in the AP (Table II.1). In the rat a progressive increase in E_2R content of the anterior pituitary occurs, reaching adult levels at 28 days of age (Kato 1980). The presence of E_2R in the posterior pituitary has not to the author's knowledge been discussed previously. Since tissue samples were so small, samples had to be pooled for one determination of E_2R, therefore, the role of PP receptors as well as the general trend to decrease from 10 to 1 fmole mg^{-1} protein at the time of first estrus remains to be confirmed.

The general lack of an increase in E_2R as found in this study is consistent with earlier results reported by Diekman and Anderson (1982a) and Diekman and Anderson (1982b). Both studies by Diekman and Anderson suggested that no increase in receptor content of the whole pituitary or whole hypothalamus took place during maturation of the gilt. Apart from the general lack of

change with age in E_2R it is not possible to compare in detail the E_2R concentrations in the present study with those reported by Diekman and Anderson (1982a, 1982b) because of differences in the assay and method of reporting the results. Diekman and Anderson (1982a, 1982b) did not use a molybdate buffer, known to prevent degradation of receptors during assay (Walters 1985) and their assay was conducted on whole hypothalamus tissue samples which did not include the preoptic area. Further, these authors reported the results as fmole receptor per mg wet weight tissue and not in the preferred conventional units of fmole per mg cytosol protein.

It is apparent that development of the gilt is different from that of the female rat with respect to development of E_2R levels in the hypothalamus and pituitary. It is possible that regions of the brain other than the hypothalamus may have more important control over gonadotropin regulation by E_2R regulated neurons. For example, in the rat large quantities of E_2R are also found in the amygdala (McEwen 1976). In addition to uptake of estradiol by receptors in this area, there also exists significant concentrations of androgen receptors. It is thought by some that uptake of steroid as androgen and intracellular conversion to estrogen may have a role to play in the change in feedback sensitivity during puberty (Kato 1980). It remains to be determined however if similar patterns of E_2R location occur in the gilt or in

fact if these regions are involved in the initiation of gonadotropin regulation or the attainment of puberty.

Table II.1 Concentrations (Mean ± SEM) of 17β-estradiol receptors (fmol mg^{-1} cytosolic protein) in the hypothalamus and pituitary of gilts from 4 to 28 wk of age[1,2]

Age (wk)	Hypothalamus			Pituitary	
	Anterior	Central	Posterior	Anterior	Posterior[3]
4	8.0 ± 2.7	5.4 ± 1.2	5.7 ± 1.1	5.2 ± 1.8	10.5
8	4.9 ± 1.6	4.9 ± 1.2	5.9 ± 0.6	6.9 ± 2.3	3.6
12	3.2 ± 0.9	10.3 ± 2.9	4.0 ± 0.7	21.2 ± 3.9	3.2
16	4.5 ± 1.4	5.2 ± 1.3	5.2 ± 0.8	11.9 ± 4.6	2.4
20	12.1 ± 2.7	7.3 ± 1.8	8.5 ± 2.0	8.5 ± 3.2	1.0
24	9.1 ± 3.2	8.8 ± 1.4	6.4 ± 2.6	11.2 ± 2.4	1.0
28	3.6 ± 0.8	5.3 ± 1.1	7.5 ± 1.8	7.8 ± 2.9	1.5
Ka[4]	4.6 ± 0.1	4.8 ± .2	4.9 ± 0.1	54.7 ± 2.7	86.6 ± 8.6

1 n=5 animals per observation except anterior pituitary at 8 wk and 20 wk where n=4.
2 No significant difference with respect to age was detected (P=0.11)
3 Tissue samples pooled from 5 animals, one assay.
4 ($x10^{8}$ $Molar^{-1}$)

Fig. II.1 The relationship between concentration of 17ß-estradiol receptors (E_2R) detected and amount of protein in the assay tube for lyophilised uterus used as the control tissue.

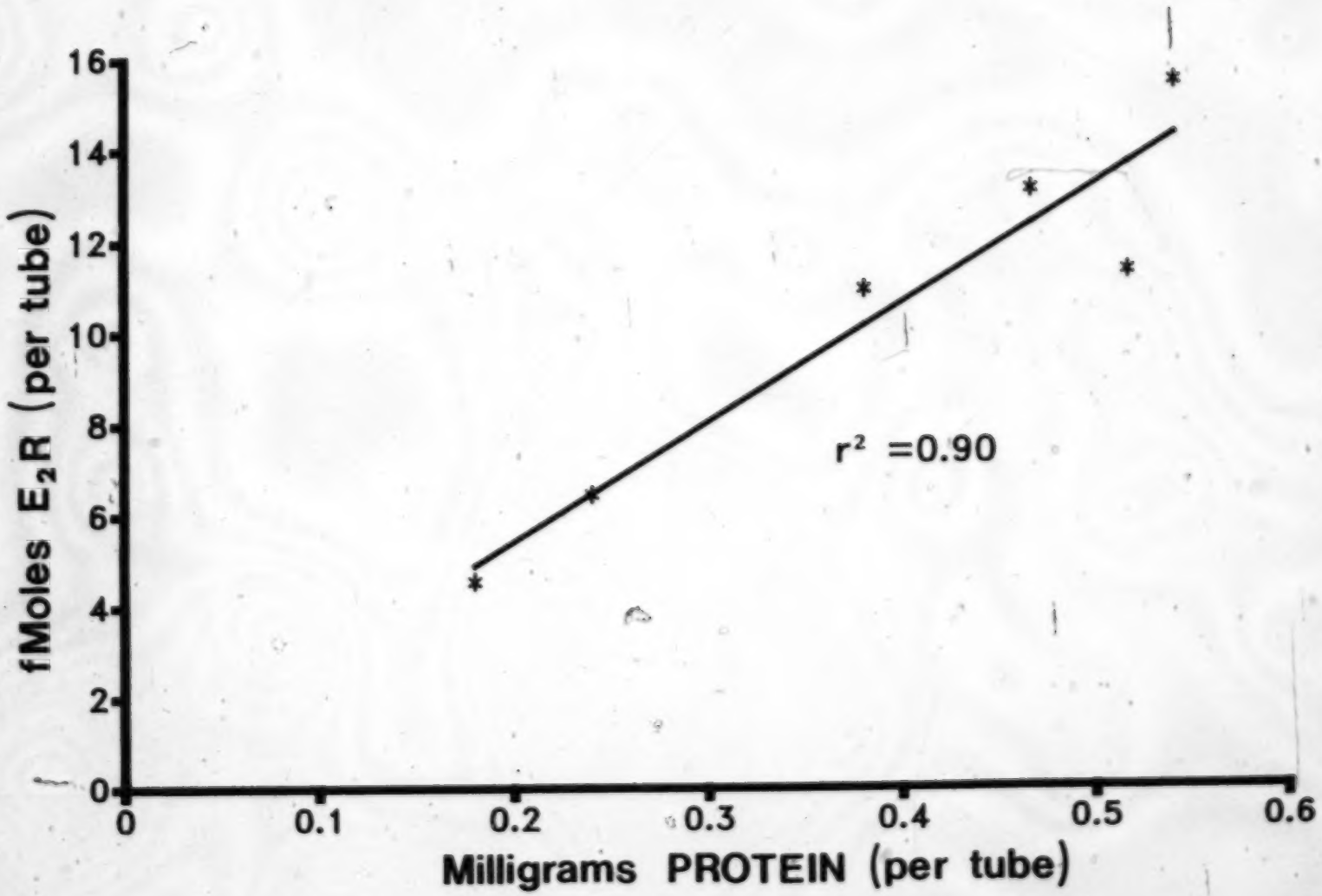

16
14
12
10
8
6
4
2
0
fMoles E_2R (per tube)
r^2 =0.90
0
0.1
0.2
0.3
0.4
0.5
0.6
Milligrams PROTEIN (per tube)

Fig. II.2 Saturation curves of the specific binding of tritiated estradiol-17ß from 0.05 to 5.0 nM by cytosol from the anterior hypothalamus (AH), central hypothalamus (CH), posterior hypothalamus (PH), anterior pituitary (AP) and posterior pituitary (PP) tissue samples.

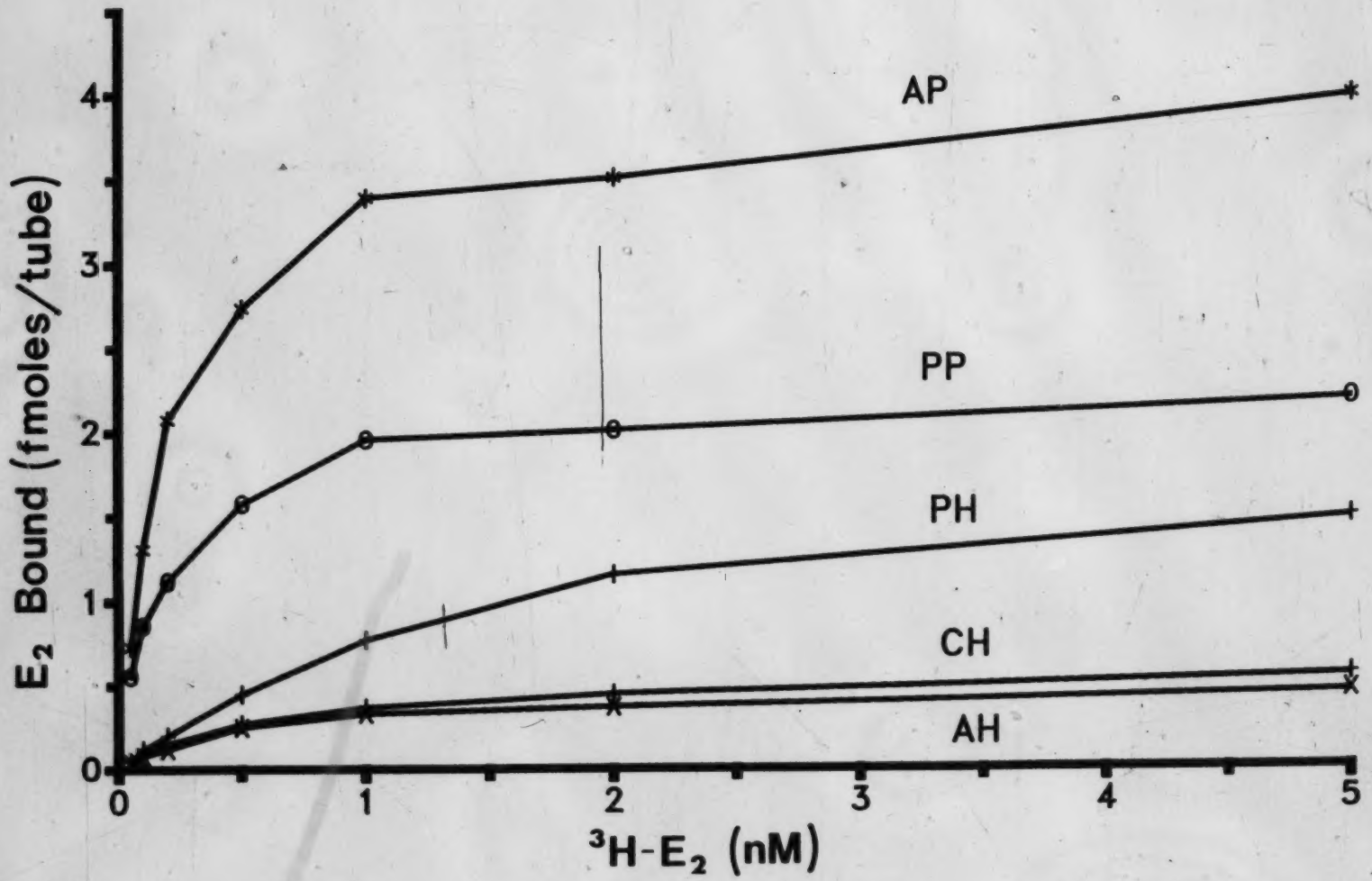
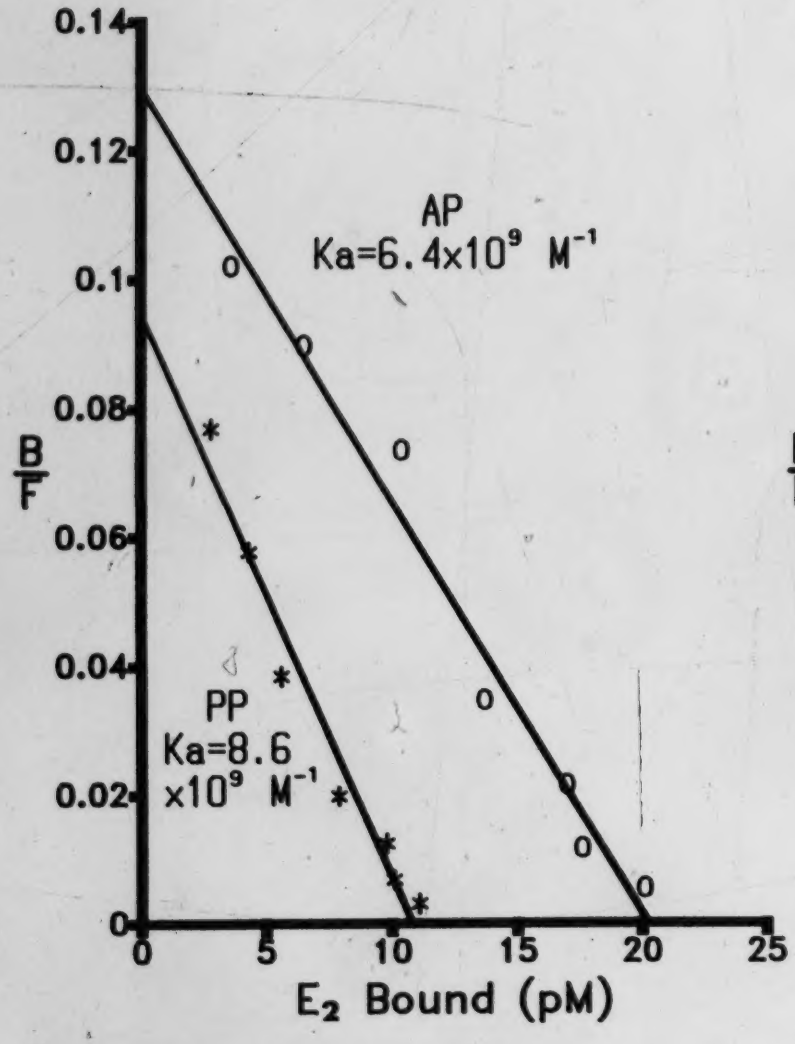
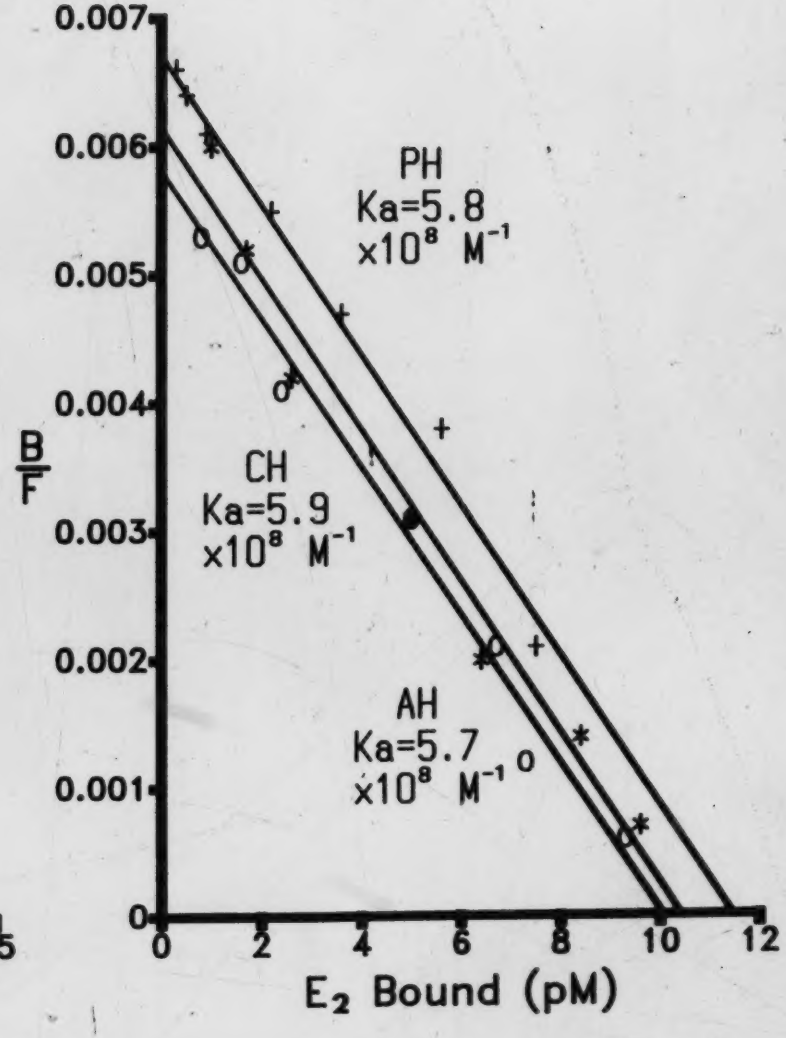

AP
PP
PH
CH
AH
E_2 Bound (fmoles/tube)
^3H-E_2 (nM)
0
1
2
3
4
5

Fig. II.3 Examples of Scatchard plots of the specifically bound to free ratio of tritiated estradiol-17ß (B/F) vs specifically bound tritiated estradiol-17ß (E_2 Bound picoMolar) for cytosol from the anterior hypothalamus (AH), central hypothalamus (CH), posterior hypothalamus (PH), anterior pituitary (AP), and posterior pituitary (PP) indicating the affinity constant (Ka) obtained by fitting a regression line through the data points.


0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
B/F
AP
Ka=6.4x10⁹ M⁻¹
PP
Ka=8.6
x10⁹ M⁻¹
0
5
10
15
20
25
E₂ Bound (pM)


0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
B/F
PH
Ka=5.8
x10⁸ M⁻¹
CH
Ka=5.9
x10⁸ M⁻¹
AH
Ka=5.7
x10⁸ M⁻¹
0
2
4
6
8
10
12
E₂ Bound (pM)

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III. DEVELOPMENT OF THE CIRCADIAN RHYTHM OF PLASMA CORTISOL IN THE GILT FROM WEANING UNTIL SEXUAL MATURITY

INTRODUCTION

Most physiological processes, including hormonal release, occur in a rhythmic fashion. In the mature adult a distinct diurnal rhythm in the release of adrenal steroids has been shown for most mammals including the mouse (Luce 1971), rat (Allen and Kendall 1967), human (Weitzman et al. 1971) and cow (Fulkerson et al. 1980). In swine a circadian rhythm has been reported for both mature sows (Bate and Hacker 1985) and boars (Fonda et al. 1981).

Although release of adrenal steroids in the adult is clearly rhythmic, development of this rhythm is a gradual process, maturing as the individual grows and matures. In the rat, plasma corticosterone concentrations are low and non-rhythmic in the neonate and gradually increase both in amount and rhythmic intensity as the animal matures (Takahashi et al. 1979). In the human a similar period of development of the rhythm takes place although baseline levels of cortisol are higher than in the rat (Franks 1967). There does not appear to be information available on the maturation of the rhythm of adrenal steroid release in swine.

Despite the fact that the exact role of the adrenal hormones in the maturation of other organ systems has not

been clearly defined there is some evidence that these hormones can affect development of the reproductive axis (Gorski and Lawton 1973). In addition, the presence of the rhythmic component of adrenal hormone output has been associated with normal estrous cycles and fertility (Paris and Ramaley 1974).

In order to study the interrelationship of the reproductive axis and adrenal axis with respect to puberty and fertility of the gilt, an understanding of the normal schedule for maturation of adrenal hormone output is required. The objective of the present study was to characterize the development of the circadian rhythm of cortisol release in the gilt from 4 wk of age to 28 wk of age.

MATERIALS AND METHODS

Animals and Experimental Procedures

A group of 20 Yorkshire x Landrace gilts were randomly selected at weaning (4 wk). Ten animals were serially blood sampled and ten animals served as controls but all animals were monitored for age at first estrus. Lighting was by means of cool white fluorescent lamps operating from 7:00 h to 17:00 h and this study took place from August to January. Animals were housed and fed as described in Chapter II.

At 4, 8, 12, 16, 20, 24 and 28 wk of age blood samples

were collected throughout a 24 h period. Because non-surgical venous cannulation was not feasible for small animals, samples at 4, 8 and 12 wk were collected every 2 h by means of suborbital sinus puncture using 18 g needles (Monoject 250, Becton-Dickinson, Rutherford, NJ, USA). At 16, 20, 24 and 28 wk of age hourly blood samples were collected by means of an indwelling vena cava cannula.

Twenty-four hours before blood sampling by cannula animals were moved to individual stalls located in the same room in which they were normally housed. Twelve hours before blood sampling a cannula (PE 90, Clay Adams) was inserted into an ear vein through a 14 g needle (Monoject 200, Becton-Dickinson) until the distal end was located in the vena cava. This procedure was accomplished without the use of anesthesia using a head gate as restraint. Heparinized saline (60 IU heparin mL^{-1} saline) was used to keep the lumen of the cannula patent. At the time of sampling a cannula extension was fitted and suspended above the animal's pen. This allowed sampling without disturbing the animal. Feed and water were provided ad libitum during the entire time the animals were confined to the individual stalls . At the end of the sampling period the cannulas were removed and the animals were returned to their original pens.

In order to ensure that blood samples taken via the suborbital sinus were comparable to samples taken via the cannula and not affected by the stress of sampling a

pretrial was conducted. Using the method described, five 16 wk old animals were cannulated and sampled via the cannula at frequent intervals starting 60 min before and ending 90 min after a blood sample was collected by the suborbital sinus method. Animals used for this pretrial were sampled for 24 h periods on at least three other occasions by the suborbital sinus method. A nose snare was used as a restraint during the suborbital sampling.

All blood samples were collected into heparinized tubes (10 IU mL^{-1} blood) and held at 4°C until separation of plasma. Centrifugation took place at 1500 x g for 30 min at 4°C and the plasma was frozen and stored at -20°C until assayed for total cortisol and non-cortisol binding globulin (non-CBG) bound cortisol.

All animals were observed daily for visual signs of estrus from 22 wk onwards. Estrus was confirmed by examination of the ovaries after slaughter, 10 d after signs of estrus.

Hormone Assays

To assay for total cortisol, 5 µL plasma samples were diluted with 500 µL Tris-gel buffer (0.05 M Tris-HCl, 0.1 M NaCl, 0.015 M sodium azide, 0.1% gelatin) and heat treated in 12 x 75 mm glass tubes for 15 min at 95°C to denature cortisol binding globulin (Forster and Dunn 1974). The antisera was diluted as recommended by the supplier (rabbit anticortisol-21-thyroglobin, Lot COR3, Miles-Yeda, Toronto, Canada) and the amount required for

each tube was added in 100 µL of Tris-gel buffer at 4°C. After incubating overnight, labelled cortisol (1,2,6,7-^{3}H hydrocortisone, 93 Ci mmole^{-1}, New England Nuclear), 14000 dpm in 100 µL Tris buffer was added and the tubes incubated overnight at 4°C. Separation of free from bound cortisol was achieved by the addition of 200 µl of 0.5% charcoal (Norit A, Fisher Scientific, Fair Lawn, NJ, USA), 0.05% Dextran (70,000 M. Wt., Sigma) in Tris-gel. Following incubation for 10 min at 4°C, the contents were centrifuged at 3000 x g for 15 min and a 500 µL aliquot of supernatant was counted in a liquid scintillation counting system.

Validation of the cortisol radioimmunoassay was as described by Chard (1982). Specificity of the assay was verified by demonstrating parallelism (P>0.5) between the standard curve and increasing concentrations (from 1 to 30 µL per tube) of pooled gilt plasma. Cross reactions of the anticortisol as reported by the supplier were: compound S, 13.6%; corticosterone, 11.7%; 17ß-OH progesterone, 9.4%; progesterone, 6.9%; deoxycorticosterone, 6.0%; testosterone, 4.0%; prednisolone, <1.0%; aldosterone and androstenedione, <0.5%; dehydroepiandrosterone, estradiol and dexamethasone, <0.01%. Accuracy, evaluated by adding known quantities of standard (hydrocortisone, Sigma) covering the full range of the standard curve (from 1 to 200 ng mL^{-1}plasma for a 5 µL sample volume) to a control

serum and determining recovery of standard was 96.7 ± 4.5%. Sensitivity of the radioimmunoassay, determined as that standard that was just different from the maximum binding tubes at the 95% confidence level was 1.0 ng mL^{-1} plasma. Precision of the assays were established by including full standard curves including pooled gilt plasma samples at 200 tube intervals in each assay. Coefficients of variation were calculated based on samples of pooled gilt plasma within one assay (intraassay) and between assays (interassay). Intraassay and interassay coefficients of variation were 6.9% and 8.9%, respectively.

Non CBG-bound cortisol was measured on five of the ten animals from which blood samples were collected using a modification of the method reported by Zhang et al. (1984). For each plasma sample, labeled cortisol, 20,000 dpm in 100 µL methanol (1,2,6,7-^{3}H hydrocortisone, 93 Ci $mmole^{-1}$, New England Nuclear, Boston, MS, USA) was added to four 12 x 75 mm glass tubes and evaporated to dryness in a vacuum oven. One pair of the tubes (total count tubes) also contained a large excess of cortisol (hydrocortisone, Sigma, St. Louis, MO, USA) (2 µg) in 100 µL of methanol which was evaporated to dryness. To each tube 200 µL of plasma sample was added and the tubes incubated at 37°C for 1 h. The CBG fraction was precipitated by adding 800 µL of a 62.5% saturated solution of ammonium sulfate at 0°C to the plasma

sample, resulting in a final concentration of 50% saturated ammonium sulfate. Tubes were immediately centrifuged at 3000 x g for 10 min at 0°C and a 500 µL aliquot of supernatant was counted in a liquid scintillation counting system with quench correction. The percentage of non-CBG bound cortisol was calculated by dividing counts in the unknown supernatant by the counts in the total count supernatant.

Statistical Analysis

Peak and trough cortisol values in the circadian rhythm profiles were compared using Student's-t test. Mean total cortisol and percent non-CBG bound cortisol were evaluated using one-way analysis of variance and the differences compared using Student-Newman-Keul's test (Steel and Torrie 1980). Mean age at first estrus as well as suborbital vs cannula cortisol values were compared using Student's-t test.

RESULTS

Results of the preliminary trial to compare blood sampling techniques showed that plasma cortisol levels did not rise significantly above baseline samples collected via the cannula until 5 min after beginning of restraint of the animal for suborbital sinus puncture (Fig. III.1). Furthermore, plasma levels returned to baseline within 30 min of the suborbital sampling. In this study all

suborbital samples were collected within 2 min of contact with the animal and at 2 h intervals. It can be concluded that suborbital sinus blood samples collected in this fashion gave levels of cortisol comparable to baseline levels obtained from samples taken by indwelling vena cava cannulas.

Twenty-four hour profiles for total and non-CBG bound cortisol are shown in Fig. III.2. In some cases sample numbers varied at different ages due to loss of cannulas or inability to recannulate some of the original ten animals sampled at 4 wk intervals. Fig. III.3 depicts mean total cortisol values subjected to 3-point moving average analysis for the same time periods. From these profiles it can be observed that at 4 wk of age no distinct diurnal variation occurs. A comparison of peak and trough values at this time indicated no difference ($P>0.05$). At 8 and 12 wk of age, profiles showed evidence of a trough forming in the late afternoon. Comparisons of peak and trough values from 8 wk of age until 28 wk of age indicated a significant difference ($P<0.05$) in every cortisol profile. The profile at 16 wk of age showed the presence of a large afternoon peak along with a late evening trough. At 20 wk of age the afternoon peak disappeared but an early morning peak and evening trough were evident. At 24 wk the formation of 2 peaks and a trough were observed but cortisol did not reach adult profiles until 28 wk of age. This adult profile was characterized by a large morning

peak, a smaller afternoon peak and an evening trough. At each time period non-CBG bound cortisol profiles closely mirrored total cortisol profiles.

Mean total cortisol and percent non-CBG bound cortisol levels averaged over 24 h for each animal are shown in Table III.1. Mean total cortisol values were higher at 8 wk, 12 wk and 24 wk compared to 4, 20 and 28 wk ($P<0.05$). However, only 16 wk non-CBG bound cortisol levels were higher than at 4, 8, 20, 24 or 28 wk ($P<0.05$).

Age at first estrus for the nonsampled control animals (203.4 $\pm$ 6.8 d,) was not different ($P=0.66$) than that of the sampled animals (199.6 $\pm$ 5.3 d).

DISCUSSION

The gradual development of the circadian rhythm of cortisol in the maturing gilt can be seen from the total cortisol profiles in Fig. III.2 and more clearly in Fig. III.3. The profiles progressed from essentially nonrhythmic at 4 wk of age, to typical adult profiles at 28 wk of age. This development of the circadian rhythm of cortisol release in the gilt appears to be different however from that in the rat where low constant levels of cortisol gradually rise to form peaks (Takahashi 1979). Development of the rhythm in the gilt is similar to development in the human where neonatal levels are initially high, although not circadian in nature,

gradually developing a rhythm by decreasing cortisol levels in the lower and trough areas of the hormone profile (Vermes et al. 1980; Franks 1967).

Although the gradual development of the circadian rhythm of cortisol release is shown in this study it has not been definitely determined what level of the hypothalamo/pituitary/adrenal axis is responsible for the rhythmic release. Studies in the rat have shown that the ability of the adrenal gland to release corticosterone in response to stimultion with ACTH as well as the ability of the anterior pituitary to secrete ACTH in response to stress occur well before a circadian rhythm in corticosterone is evident (Allen and Kendall 1967). This suggests that some component of the central nervous system is responsible for the rhythm of adrenal steroid release and its progressive development. In confirmation of this, it has been shown that the content of CRF in the rat hypothalamus shows diurnal rhythmicity (Sadow 1979). This suggests that maturation of the circadian rhythm of adrenal steroid release actually reflects maturation of those parts of the central nervous system controlling the hypothalamo/pituitary/adrenal axis.

Cortisol in plasma exists in three major fractions. It is either bound to CBG, bound to albumin or unbound (Westphal 1975). The CBG bound fraction is characterized by high affinity but low capacity binding whereas the albumin bound fraction is characterized by relatively low.

affinity but larger capacity to bind cortisol. Based upon a variety of experimental evidence it is generally considered that the level of CBG regulates the free hormone concentration whereas the albumin bound fraction can be considered part of the free steroid pool. This is because the binding of cortisol to albumin has low affinity and thus can rapidly dissociate, supplementing the free pool of hormone (Westphal 1971).

Since 24 h profiles of non-CBG bound cortisol closely mirrored 24 h profiles of total cortisol (Fig. III.2) and since mean non-CBG bound cortisol levels did not vary greatly during age at development except at 16 wk (Table III.1), the amount of non-CBG bound cortisol was very nearly a constant percentage of total cortisol. Furthermore, CBG bound cortisol accounted for such a small percentage of total cortisol that in the gilt, CBG probably has little role in dampening fluctuations in unbound cortisol concentration (Pugeat et al. 1984). The low percentage of CBG-bound cortisol as determined in this study agrees favourably with the low cortisol binding capacity in serum of swine described previously (Westphal 1971). In swine cortisol binding capacity is about one fifth that reported for the human (Westphal 1971). It therefore appears that in swine total cortisol concentration, and not the free vs bound ratio, is the prime determinant of the physiological effects of this steroid.

The results shown in Table III.1 indicate that there were two distinct periods of time at which mean total cortisol levels were high. Cortisol levels were high at 8 to 12 wk of age, just prior to the age at which basal levels of gonadotropins are also found to increase (Chapter V; Diekman et al. 1983). Mean cortisol levels rise again at 24 wk of age. This occurs just before first estrus at 28 wk, at which time LH and FSH increase in a cyclic manner which is repeated with each subsequent estrous cycle.

These elevated mean cortisol levels have not, to the authors knowledge been reported previously although there is indication in the literature that a similar situation may occur in the human. High plasma concentrations of cortisol accompanied by a diurnal variation have been reported to occur at 3 y of age in the human (Franks 1967). In fact, these high levels have been interpreted as the final maturation of adrenal steroid output. In the present study (Fig. III.3) it would also seem that full development of adult profiles of cortisol is reached at 12 wk, however, further sampling at 16, 20 and 24 wk point to only a transitory increase in the cortisol profile. This same situation cannot be confirmed in the human since there are no studies which have adequately documented the development of the circadian rhythm of cortisol between 3 y and adulthood.

The pattern of a transitory increase in basal levels

of gonadotropins in the juvenile mammal as well as other endocrine events that cannot be explained fully by the traditional theories of sexual maturation have recently been interpreted in a different manner (Lehrer 1985). This newest hypothesis suggests that the interaction of two rhythms in the brain, one, cued by the circadian variation in the environment and the other, an endogenous age dependent rhythm originating in the arcuate nucleus, regulate reproductive function through regulation of hormonal release from the hypothalamus. The pattern of transitory increase of both gonadotropins and cortisol in the 12 wk old gilt, and then again at the time of sexual maturity can both be explained by the model suggested by Lehrer (1985).

The possibility that maturation of the adrenal axis could be controlled by the interactions of endogenous and environmentally cued rhythmic pathways has not been suggested before. It is possible that in an extension of Lehrer's model a different set of rhythmic processes may control the timing of events in the adrenal axis compared to the reproductive axis or it may be that the same basic rhythmic processes have a more general role in the timing of events in these and other endocrine axes. If the latter hypothesis is confirmed, our understanding of the interaction and relationship between the reproductive axis and adrenal axis or indeed other endocrine axes would be much enhanced.

Table III.1 Mean values (± SEM) for total and non-cortisol binding globulin (CBG) bound cortisol for gilts aged 4 to 28 wk

Age (wk)	n=	Total Cortisol ($ng\ mL^{-1}$)	n=	Non-CBG Cortisol (percent)
4	10	25.3 ± 2.2 b	5	72.2 ± 1.3 bc
8	10	37.4 ± 4.1 ac	5	69.4 ± 1.6 bc
12	10	40.1 ± 3.5 ac	5	82.4 ± 4.1 ac
16	7	26.8 ± 2.8 bc	5	87.9 ± 3.3 a
20	9	20.6 ± 2.6 b	5	74.2 ± 4.9 bc
24	6	38.3 ± 3.6 ac	5	70.7 ± 4.9 bc
28	7	24.8 ± 2.9 b	5	71.0 ± 2.3 bc

a,b,c Means in the same column without a common letter differ ($P<0.05$).

Fig. III.1 Effect of suborbital sinus puncture sampling on total plasma cortisol concentrations in 16 wk old gilts. Values reflect mean ± SEM of 5 animals. Cannula samples that differ from the suborbital sample are denoted by the asterisks.

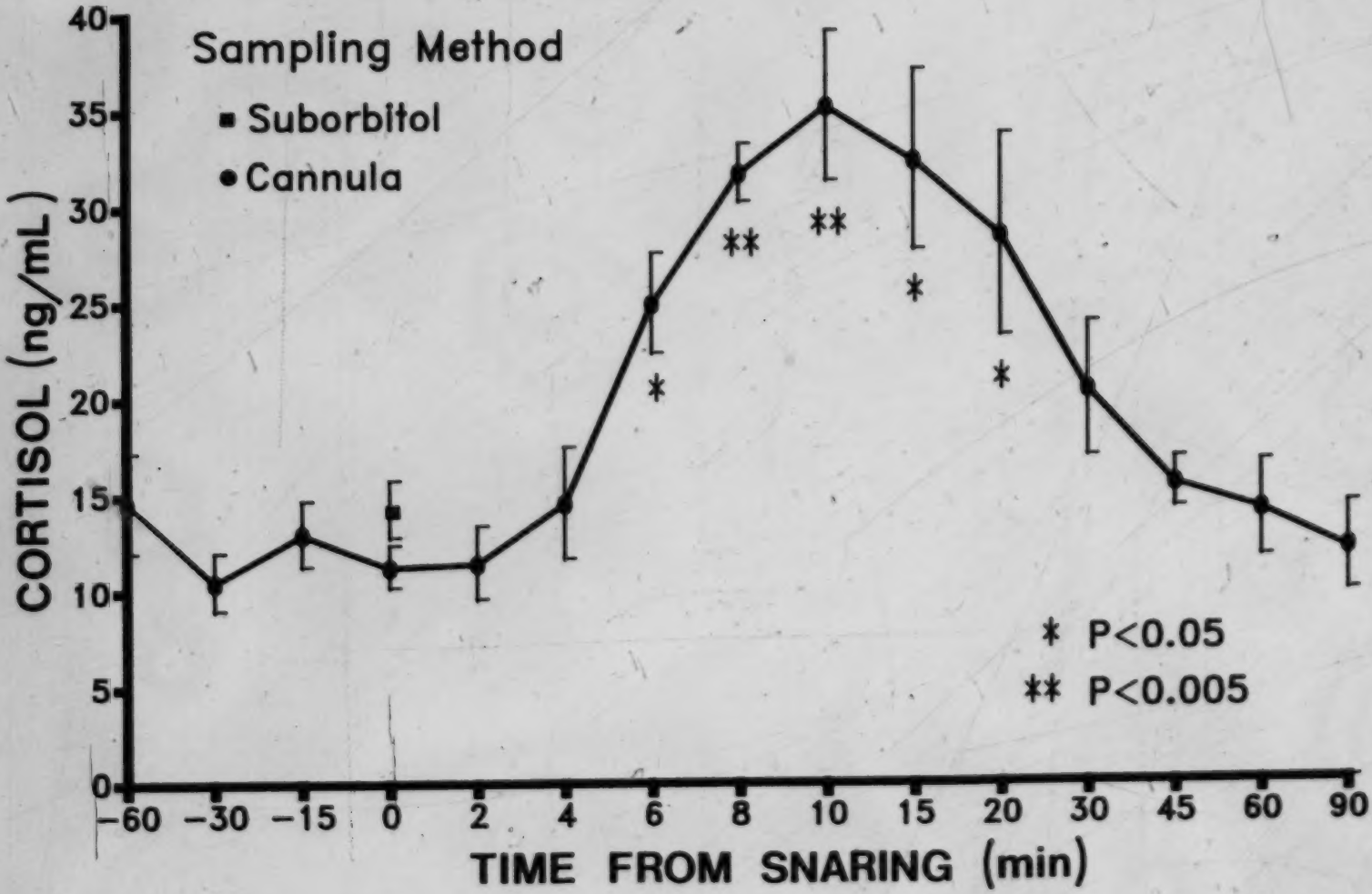

Sampling Method
■ Suborbitol
● Cannula
CORTISOL (ng/mL)
40
35
30
25
20
15
10
5
0
−60 −30 −15 0 2 4 6 8 10 15 20 30 45 60 90
TIME FROM SNARING (min)
* P<0.05
** P<0.005

Fig. III.2 Twenty-four hour profiles of mean (± SEM) total and non-cortisol binding globulin (non-CBG) bound cortisol in gilts from 4 to 28 weeks of age (n=10 for wk 4,8,12; n=7 for wk 16; n=9 for wk 20; n=6 for wk 24; n=8 for wk 28).

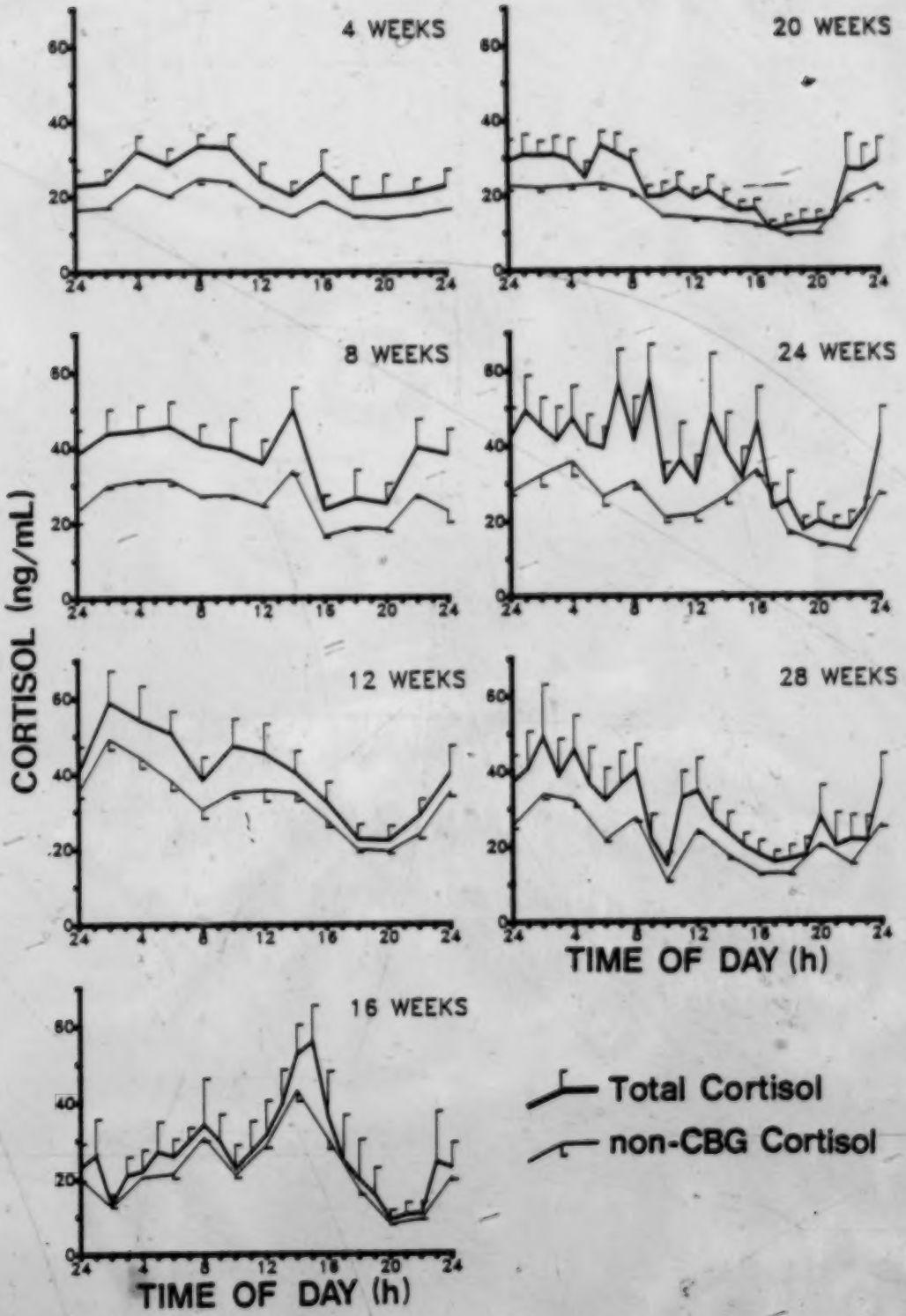
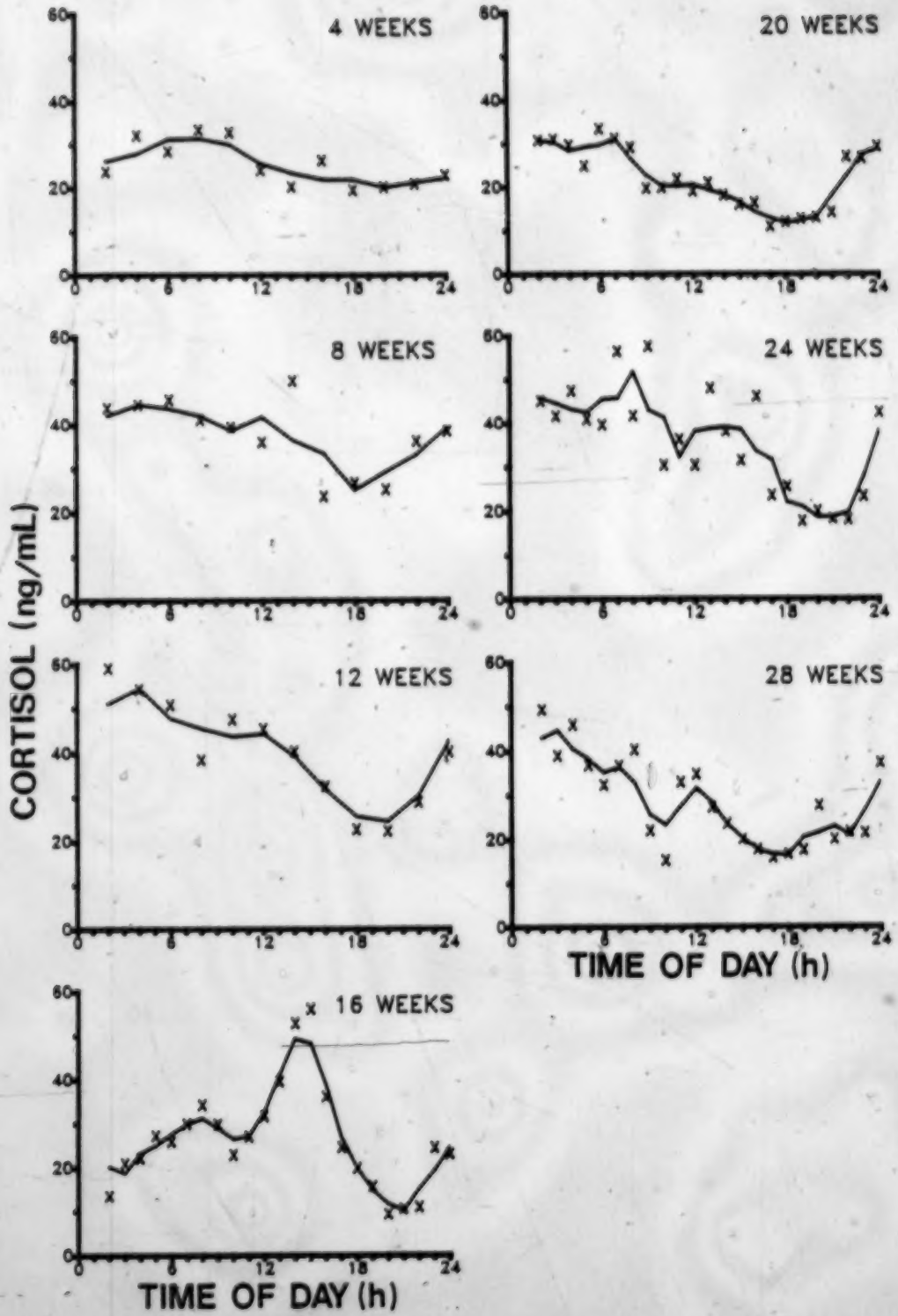

4 WEEKS
20 WEEKS
8 WEEKS
24 WEEKS
12 WEEKS
28 WEEKS
16 WEEKS
CORTISOL (ng/mL)
TIME OF DAY (h)
TIME OF DAY (h)
Total Cortisol
non-CBG Cortisol

Fig. III.3 Three-point moving average, 24 h profiles of total plasma cortisol in gilts from 4 to 28 weeks of age and the actual mean values denoted by the crosses.


4 WEEKS
20 WEEKS
8 WEEKS
24 WEEKS
12 WEEKS
28 WEEKS
16 WEEKS
CORTISOL (ng/mL)
TIME OF DAY (h)
TIME OF DAY (h)

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IV. EFFECT OF SHORT TERM AND LONG TERM STIMULATION OF THE ADRENAL AXIS BY ACTH ON PLASMA LH AND ON SUBSEQUENT PLASMA PROFILES OF CORTISOL IN THE GILT

INTRODUCTION

Stressing an animal results in the activation of a variety of behavioral, autonomic and neuroendocrine responses. Even though the autonomic nervous system plays an important role during acute stress it is probably the neuroendocrine system that has the greatest potential for altering reproduction over the long term (Moberg 1985a). Probably the most important neuroendocrine system to be stimulated by stress is the hypothalamo/pituitary/adrenal axis. In fact, the adrenal-cortical response to stress, measured by an increase in circulating titers of corticosteroids is used by many researchers in the field of stress physiology as proof (a priori) that an animal has been stressed (Moberg 1985b).

It is generally considered that ACTH activation of the adrenal axis will result in negative effects on reproduction. Liptrap (1970) reported that administration of ACTH delayed estrus, shortened the duration of estrus and interferred with ovulation in the sow. Experiments with cattle have suggested that adrenal steroids act to depress both the basal LH release and the estradiol stimulated LH surge (Li and Wagner 1983a).

There are indications however, that stressors, possibly acting through the adrenal axis, may be important in the early induction of puberty. In the rat, adrenalectomy at critical stages of maturation results in a significant delay in age at vaginal opening (Gorski and Lawton 1973). Paris and Ramaley (1974) in experiments with mice have observed that when older, noncycling females were stressed by immobilization there was a restoration of normal estrous cycles, ovulation and fertility. In swine, the application of stressors such as transport or mixing, have been shown to significantly decrease age at first estrus (Thibault et al. 1966; Signoret 1972; Bourn et al. 1974).

It is not clear from those studies reporting a positive effect of stress and ACTH on reproduction whether the high plasma concentrations of corticosteroids had direct effects or whether the effects resulted from a subsequent decrease in basal corticosteroid concentrations after the stimulus was removed. Although feedback inhibition of basal ACTH has been reported for several species including the dog (Keller-Wood et al. 1983) and the human (Reader et al. 1982) there is no information available concerning a similar effect in swine.

This study was conducted to determine whether activation of the adrenal axis by ACTH would have positive or negative effects on basal LH concentrations and on the estradiol stimulated LH surge. In addition, the effects of

stimulation of the adrenal axis by exogenous ACTH on the subsequent plasma concentrations of cortisol was examined to determine if feedback inhibition could have a role to play in the stress induction of estrus in the prepubertal gilt.

MATERIALS AND METHODS

A total of 56 Yorkshire x Landrace gilts, selected at weaning, were randomly assigned to one of four treatments. Two short term treatments consisted of 14 animals each, receiving either one injection of 40 IU ACTH (Sigma, St. Lous, MO, USA. Grade II, 85 IU mg^{-1} dissolved in sterile 0.9% saline) (one ACTH) or one injection of vehicle (one saline). The two long term treatments consisted of 14 animals each receiving either 10 injections of 40 IU ACTH (ten ACTH) or 10 injections of vehicle only (ten saline). All injections were administered intra-muscularly in the neck region at 7:00 h and in the long term treatments injections were given 24 h apart. Treatment injections were given such that the last injection was administered on day 140 of age.

The ACTH (Porcine, grade II, 85 IU mg^{-1}, Sigma, St. Louis, MO, USA) was prepared in advance by dissolving in sterile saline and dispensing into single dose vials 40 IU ACTH (40 IU mL^{-1}). Vials containing ACTH or the same volume of saline vehicle were then snap frozen in a liquid

nitrogen/methanol freezing bath and stored at -20°C until just before use.

Prior to 139 d of age all animals were raised in straw littered pens, under confined, heated housing arrangements. Composition and analysis of diets fed are listed in the Appendix (Table 1, Table 2 and Table 3). Lighting was by means of cool white fluorescent lamps illuminated from 7:00 h to 17:00 h. On day 139 all animals were implanted with an ear vein cannula terminating in the vena cava (Polyvinylchloride tubing, SV. 61, Dural Plastics, Dural, N.S.W. Australia). The cannula was fixed to the animal's ear and back with tag cement and adhesive tape. Cannulation was conducted without anesthesia, with the use of headgate restraint only. The lumen of the cannula was kept patent with a heparin saline solution (60 IU heparin mL^{-1} sterile, isotonic saline). Following cannulation animals were moved to individual pens, designed to restrict movement but in which the animals could stand and lie at will. Prior to blood sampling a 1 m cannula extension was fitted and suspended above the animal's pen. This allowed sampling without disturbing the animal. Throughout the experiment (including blood sampling) fresh water and feed was available ad libitum.

On day 140, at least 12 h after cannulation, blood sampling commenced at 1 h intervals at 2:00 h and continued until 20:00 h on day 141 of age. These samples, after assaying for total plasma cortisol were used to

determine the effects of ACTH on subsequent concentrations of plasma cortisol. Additional blood samples, collected on day 140 at 15 min intervals for 4 h (8:00 h - 12:00 h) were used to monitor basal levels of LH. At 20:00 on day 141 all animals received an intra-muscular injection of 17ß-estradiol-3-benzoate (20 µg kg^{-1} live weight) in corn oil. Samples of blood were collected for an additional 72 h at 6 h intervals and after assaying for LH used to monitor the effects of ACTH on the estradiol induced LH surge. All blood samples were collected into heparinized tubes (10 IU mL^{-1} blood) and kept at 4°C for no more than 12 h until centrifugation at 3000 x g for 30 min at 4°C. Plasma samples were then frozen and stored at -20°C until assayed for hormone content.

Hormone Assays

The radioimmunoassay validation and procedure for plasma cortisol using rabbit anticortisol-21-thyroglobin (Lot COR3, Miles-Yeda Ltd. Toronto, Ontario, Canada) has been described previously (Chapter III). Intraassay and interassay coefficients of variation were 5.2% and 9.4% respectively.

Concentrations of LH were determined by radioimmunoassay using rabbit-antibovine-LH serum as described by Kraeling et al. (1982). The validation procedure was as described for cortisol radioimmunoassay in Chapter III. Assay sensitivity was 0.2 ng mL^{-1} plasma and recovery of purified standard added to control plasma

was 93.0 ± 1.8%. The intraassay and interassay coefficients of variation were 5.4% and 5.8%, respectively. Dose response curves for pooled porcine plasma and increasing concentrations of porcine LH (LER-786-3) were parallel (P>0.5).

Estradiol-17ß was measured using radioimmunoassay procedures described by Wettemann et al. (1972). The validation procedure was as described in Chapter III for cortisol. Cross reactions for the rabbit antiserum against estradiol-17ß (E-26-47, Endocrine Sciences, Tarzana, CA) as reported by the supplier were: estrone, 1.3%; estriol, 0.6%; 17alpha-estradiol, 0.1%; cortisol, corticosterone, progesterone, testosterone, all <0.01%. Sensitivity was 7.5 pg mL^{-1} plasma and assay recovery was 105.0 ± 5.7% (mean ± SEM). Recovery from the solvent used for extraction (diethyl ether) was consistently 90% and this value was applied as a correction factor in determining hormone concentrations. Intraassay and interassay coefficients of variation were 6.1% and 13.9% respectively. Parallelism between the dose response curves for pooled porcine plasma and increasing concentrations of estradiol-17ß standard (Sigma) was demonstrated (P>0.5).

Statistical Analysis

The area under the cortisol profiles as well as average plasma cortisol concentrations on both Day 1 (first day of blood sampling and day of last ACTH or

saline injection) and Day 2 (second day of blood sampling and day following last injection) were analysed by three way analysis of variance with one repeated measure (Steel and Torrie 1980).

Pulsatile LH release was evaluated by defining a pulse as the peak value preceeded and followed by a nadir with the peak at least greater than the sensitivity of the LH assay. Time between pulses was then defined as the average time between successive pulses of LH. Frequency of pulsatile release was determined by obtaining the average number of LH pulses per 4 h of collected samples. Mean LH level was determined by obtaining the average of all LH concentrations during the 4 h sampling window.

The LH surge was defined as the peak value obtained, greater than two standard deviations from the mean baseline.

Treatment means for the effects of ACTH on plasma LH were compared to their respective controls by Students' t-test. Interactions between long and short term and saline vs ACTH were assessed by means of two way analysis of variance (Steele and Torrie 1980). Differences were considered nonsignificant if $P>0.05$.

RESULTS

Plasma cortisol profiles for Day 1 and Day 2 are depicted in Fig IV.1 and in Fig. IV.2 for short term (one

injection) and for long term (ten injections) treatments respectively. The area under the cortisol profiles from 2:00 to 20:00 h of both Day 1 and Day 2 as well as mean plasma cortisol concentrations for the same time intervals are presented in Table IV.1.

Statistical analysis of mean cortisol levels indicated that ACTH injection significantly raised mean cortisol levels compared to saline injection (41.1 $\pm$ 2.5 vs 31.3 $\pm$ 1.8 ng mL^{-1}, one and ten ACTH pooled vs one and ten saline pooled, P=0.006). There was no effect however of one vs ten injections (39.1 $\pm$ 2.0 vs 33.3 $\pm$ 2.4 ng mL^{-1}, one ACTH and one saline pooled vs ten ACTH and ten saline pooled, P=0.095). Analysis of the interaction of Day 1 vs Day 2 with the effect of ACTH or saline indicated that only ACTH on Day 1 increased mean cortisol levels when compared to ACTH, Day 2 or saline injection, Day 1 or Day 2 (52.48 $\pm$ 3.3 vs 29.7 $\pm$ 2.3, 32.9 $\pm$ 2.8 or 29.7 $\pm$ 2.2 ng mL^{-1} respectively, P=0.001).

The area under the cortisol profile was significantly increased as a result of ACTH treatment compared to saline (750.5 $\pm$ 46.2 vs 568.5 $\pm$ 33.5 h ng mL^{-1}, one and ten ACTH pooled vs one and ten saline pooled, P=0.005). However, there was no difference between one injection and ten injections (711.9 $\pm$ 3.8 vs 607.1 $\pm$ 44.8 h ng mL^{-1}, one ACTH and one saline pooled vs ten ACTH and ten saline pooled, P=0.097). Analysis of the interaction of Day 1 vs Day 2 with the effect of ACTH vs saline indicated that

only ACTH administration on Day 1 of injection increased the area under the profile compared to Day 2 or to saline injection, Day 1 or Day 2 (966.7 $\pm$ 60.5 vs 534.2 $\pm$ 39.7, 597.9 $\pm$ 51.6 or 539.1 $\pm$ 42.8 h ng mL^{-1}, P=0.001).

Basal LH release parameters for both short and long term ACTH stimulation as well as respective controls are summarized in Table IV.2. Injection of ACTH resulted in differences of frequency of LH pulses (per 4 h) that just reached significance for the short term stimulated group (4.9 $\pm$ 0.3 vs 4.1 $\pm$ 0.3, P=0.05) but not for the long term stimulated group (4.5 $\pm$ 0.3 vs 4.0 $\pm$ 0.3, P=0.30). An example of a pulsatile LH release profile for one animal is depicted in Fig. IV.3. No differences were detected in the time between LH pulses or mean LH levels. Analysis of variance failed to detect any interactions between number of injections and substance injected (P>0.05) for any parameter monitored for basal LH characteristics.

The parameters monitored with respect to the estradiol induced LH surge are presented in Table IV.3. Since there were no significant differences (P>0.05) in any of these parameters LH surge profiles were pooled across all treatments and the means shown in Fig. IV.4. The plasma estradiol levels reached in response to the injection of exogenous estradiol-3-benzoate are also shown in Fig. IV.4.

DISCUSSION

One possible explanation consistent with both negative and positive effects of activation of the adrenal axis on puberty could involve the phenomena of corticosteroid feedback inhibition of ACTH release in response to stimulation of the hypothalamo/pituitary/adrenal axis (Dallman 1979). The sites of negative feedback of corticosteroids on ACTH release are reported to include the anterior pituitary, hypothalamus and neural inputs to the hypothalamus (Keller-Wood and Dallman 1984). If these sites of feedback inhibition were involved in regulating basal corticosteroid release, the immediate effect of stress would be to increase corticosteroid output resulting in negative effects on reproductive function. However the long term effects of elevated plasma cortisol concentrations would be to decrease subsequent basal levels of ACTH release resulting in a temporary decrease in basal plasma levels of corticosteroids. These decreased concentrations of circulating adrenal hormones would then allow reproductive responses, if restricted by normal basal levels of corticosteroids to, escape restriction temporarily. If this were to occur during the prepubertal period in the gilt as a result of stress (Signoret 1972; Bourn et al. 1974), this period of escape of gonadotropin inhibition might be heightened to a point where follicular development is enhanced enough to sustain positive

feedback of estrogen resulting in first ovulation. It has been reported by Liptrap and Raeside (1975) that after ACTH administration to boars, a temporary adrenal-mediated increase in circulating levels of testosterone occurs.

In order to support the hypothesis that stress may exert temporary positive effects on reproduction through a decrease in circulating levels of corticosteroids it is necessary to demonstrate that acute activation of the adrenal axis is followed by a subsequent decrease in basal levels of serum adrenal steroids. From the results of the present study it was observed that only ACTH administration and not saline was active in increasing serum levels of cortisol (Fig. IV.1, Fig. IV.2 and Table IV.1). However, this increase in cortisol level occurred only immediately after ACTH injection and did not affect subsequent plasma concentrations of cortisol. The high levels of cortisol of at least 140 ng mL^{-1} in response to ACTH injection returned to baseline within 6 h and did not diminish subsequent cortisol release either on Day 1 or on Day 2 (Fig. IV.1 and Fig. IV.2). This is also indicated by a true increase in total area under the cortisol profile on Day 1 only (Table IV.1). On Day 2 normal circadian profiles of cortisol were observed similar to that reported in Chapter III for unstimulated gilts. Whether output of other adrenal steroids was altered as observed in rabbits after prolonged ACTH administration (Ganjam et al. 1972) is not known.

Although the present study failed to show any effects of ACTH on subsequent basal concentrations of plasma cortisol when sampled following 1 d or 10 d of ACTH administration it is possible that the administration of ACTH in a continuous rather than a single dose regime could have had a different effect. Alternatively, it may not be possible to achieve inhibition of plasma adrenal steroid levels following an acute increase due to injection of ACTH. It has been difficult to demonstrate inhibition of basal ACTH release when physiological concentrations of glucocorticoids have been employed (Keller-Wood and Dallman 1984). Only supraphysiological doses of corticosterone or dexamethasone were effective in reducing basal ACTH release in vitro (Kraicer and Milligan 1970) and in vivo there is much less effect on basal ACTH release than on stimulated ACTH release (Keller-Wood et al. 1983).

In contrast to the effects of administration of corticosteroids, prolonged exposure to corticotropin releasing factor (CRF) results in decreased responsiveness to CRF itself, expressed as a decrease in ACTH release from the pituitary (Rivier and Plotsky 1986). This phenomenon probably involves loss of receptor sites mediating the effects of CRF. The result of loss of receptors would be a desensitization of cells in the pituitary to further hormonal stimuation. This action, called "down-regulation", has been reported previously for

both steroid hormones (Cidlowski and Cidlowski 1981) and for a variety of protein hormones (Catt et al. 1979) but not to the authors knowledge for CRF. If down-regulation were to occur at one step prior to ACTH's actions, because of the cascade effect inherent in the hypothalamo/pituitary/adrenal axis the effect on decreasing corticosteroid release would probably be greater than if down-regulation of ACTH receptors in the adrenal gland were to occur. Perhaps if this experiment was to be repeated but with administration of CRF in place of ACTH, a significant effect on subsequent basal concentrations of plasma cortisol might have been observed. The physiological release of cortisol, either unstimulated (natural circadian profile) or stimulated in response to restraint, shows a time of marked release resulting in high levels followed by a return to baseline (Chapter III). High, constant levels of stimulated or injected corticosteroids are not considered physiological (Munck et al. 1984). Barb et al. (1982) reported that ACTH (100 IU twice daily in gelatin) or hydrocortisone acetate (in oil) blocked ovulation and/or suppressed estrus in gilts. From the cortisol profiles presented in their study it was clear that the gilts were subjected to abnormal patterns and concentrations of ACTH and corticosteroids. The effect of numerous injections of ACTH, for example, 80 IU three times a day for several days as reported by Liptrap (1970) would probably result in constant, high

levels of adrenal hormone output. This profile would not likely mimic profiles typical of classical types of stress such as transport, mixing or boar exposure which are known to influence the induction of puberty in the young gilt (Thibault et al. 1966).

Esbenshade and Day (1981) reported that in a study in which 80 IU ACTH in saline were administered to gilts the incidence of estrus was 6/6 compared to 4/6 for the controls. Although the latter study was limited by the small animal numbers and the absence of data on hormonal patterns, it does indicate that the effect of ACTH on the reproductive response of gilts may not necessarily be negative.

From the results of the present study it can be seen that stimulation of the adrenal axis for 1 d or for 10 d by ACTH at the level reported here did not exert negative effects on either basal LH release or on the estradiol induced LH surge. Therefore, the occurance of any negative effect of ACTH stimulation on estrus and ovulation, as suggested by Li and Wagner (1983a) for heifers may only be present in the mature, cycling female. There are indications that this is so since Li and Wagner (1983b) suggest that the major effect of ACTH or hydrocortisone succinate treatment was to reduce luteal development expressed as a low or inconsistent rise in plasma progesterone concentration. In their study ACTH or corticosteroid treatment of heifers had little effect on

basal LH levels or on LH surges (Li and Wagner 1983b). It would seem that if the effect of ACTH was restricted to alterations of luteal development and not to follicular development that it would have little role to play in the mechanism of induction of first estrus in gilts.

Even though no negative effect of ACTH stimulation of on LH release was detected in the present study, a positive effect could have been exerted at a level of the hypothalamo/pituitary/gonadal axis other than LH release. A likely candidate for this affect is FSH release. In vivo studies in rats have shown the differential regulation of FSH by both an LHRH dependent and an LHRH independent mechanism (De Paolo 1985). In addition, in vitro studies with rat pituitary cells have shown that while glucocorticoids exert only a very slight suppression of basal LH secretion, they act to increase FSH synthesis (Suter and Schwartz 1985).

In conclusion, repeated daily stimulation of gilts with exogenous ACTH did not result in a diminished plasma cortisol response or altered subsequent basal levels of plasma cortisol. Therefore no evidence was obtained to suggest that elevated cortisol levels exert long term negative feedback effects on regulatory sites of basal corticosteroid release in the gilt. In addition corticosteroids, released in response to activation of the adrenal axis by ACTH at the levels used in this study appear to have little effect on basal LH level or on the

estradiol induced LH surge in the prepubertal gilt. A possible effect of ACTH on FSH however cannot be ruled out.

Table IV.1 Effect of short term (one injection) and long term (ten injections) administration to 140 d old gilts of either saline or ACTH on mean plasma concentrations of cortisol and the area under cortisol profiles from 2:00 h to 20:00 h on Day 1 and Day 2[1]

Day[2]	Treatment	Mean Cortisol ($ng\ mL^{-1}$)	Area ($h\ ng\ mL^{-1}$)
Day 1	One Saline	35.5 ± 2.9[3]	645.8 ± 53.1
	One ACTH	53.0 ± 5.0	979.4 ± 94.4
	Ten Saline	30.3 ± 5.3	549.9 ± 95.8
	Ten ACTH	52.0 ± 4.3	954.1 ± 79.3
Day 2	One Saline	34.3 ± 3.0	619.4 ± 54.5
	One ACTH	33.5 ± 2.8	602.9 ± 49.6
	Ten Saline	25.2 ± 3.5	458.8 ± 65.5
	Ten ACTH	25.8 ± 3.1	465.5 ± 58.0

[1] n=14 animals per treatment.

[2] Day 1 = day of only or last injection,
Day 2 = day after only or last injection.

[3] Mean ± SEM.

Table IV.2 Effect of injection of 40 IU ACTH on the mean plasma LH concentration, frequency of LH pulses and time between LH pulses in 140 d old gilts[1,2].

	One Injection			Ten Injection[4]		
Parameter	Saline	ACTH	Signif.[3]	Saline	ACTH	Signif.
Freq. of LH pulses (pulses/4 h)	4.1 ± 0.3	4.9 ± 0.3	P=0.05	4.0 ± 0.3	4.5 ± 0.3	NS
Time between pulses (min)	53.0 ± 3.9	48.4 ± 3.2	NS	58.2 ± 4.3	52.9 ± 4.7	NS
Mean LH (ng mL^{-1})	1.47 ± 0.06	1.60 ± 0.09	NS	1.41 ± 0.1	1.31 ± .03	NS

[1] n=14 animals per treatment for a total of 56.

[2] Mean ± SEM.

[3] NS = no significant difference (P>0.05).

[4] 40 IU ACTH administered 24 h apart.

Table IV.3 Effect of injection of 40 IU ACTH on the estradiol-17β induced LH surge characteristics, time from estradiol injection to peak LH and peak LH level in gilts of 140 d of age[1,2]

Parameter	One Injection			Ten Injections[4]		
	Saline	ACTH	Signif.[3]	Saline	ACTH	Signif.
Time of LH surge (h)	54.5 ± 1.6	57.2 ± 1.3	NS	53.1 ± 1.6	53.1 ± 1.5	NS
Peak LH level reached ($ng\ mL^{-1}$)	15.8 ± 2.0	14.4 ± 1.8	NS	15.0 ± 2.5	14.0 ± 1.5	NS

[1] Mean ± SEM.

[2] n=13 animals each for one saline and one ACTH and n=14 animals each for ten saline and ten ACTH.

[3] NS = no statistical difference ($P>0.05$).

[4] 40 IU ACTH administered 24 h apart.

Fig. IV.1 Effect of one injection of ACTH (one ACTH) or saline (one saline) at 7:00 h on day 140 of age on plasma cortisol concentrations from 2:00 h on day 140 of age (Day 1) to 20:00 h on day 141 of age (Day 2) in gilts.

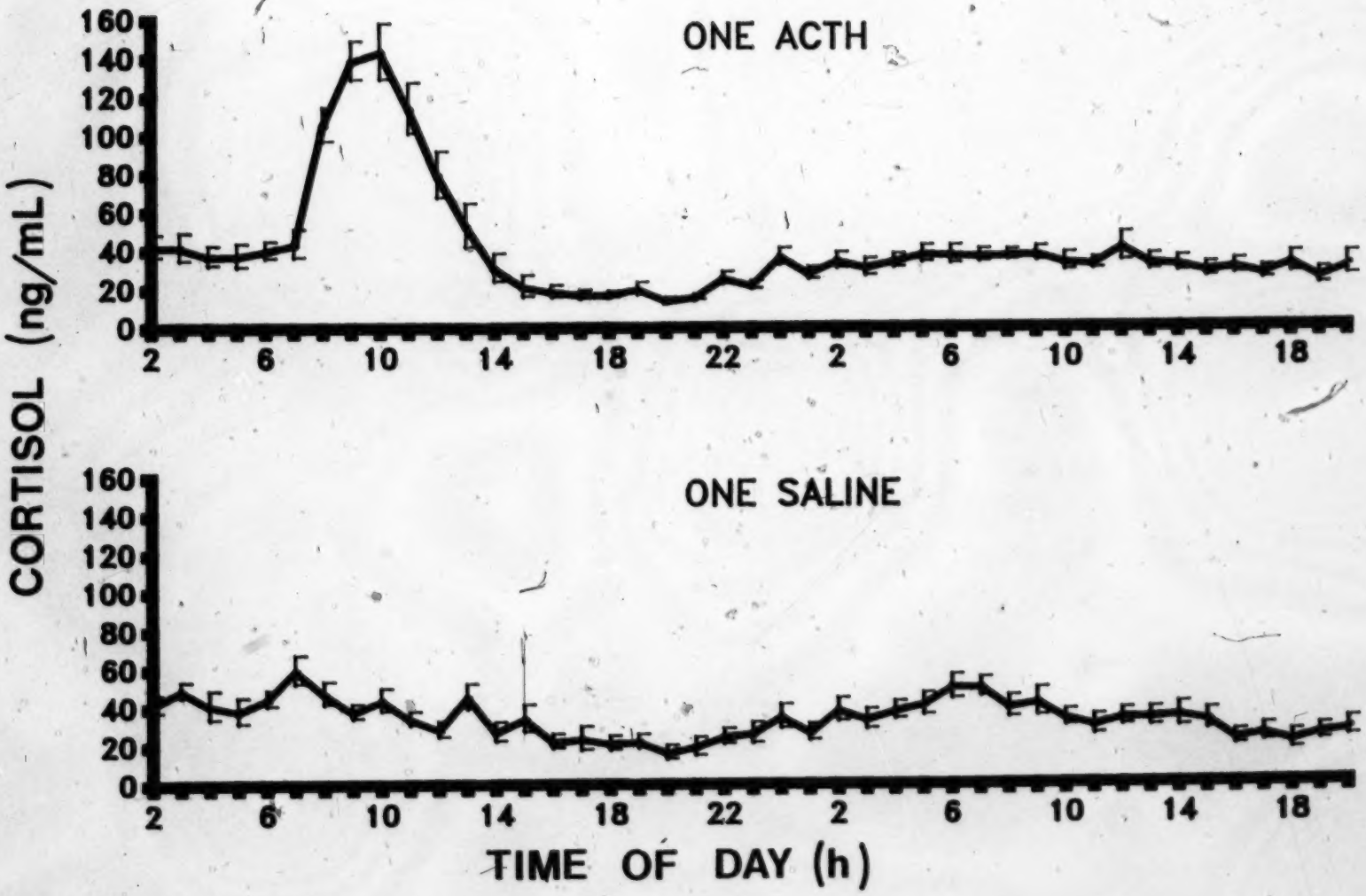

ONE ACTH
ONE SALINE
CORTISOL (ng/mL)
160
140
120
100
80
60
40
20
0
2
6
10
14
18
22
2
6
10
14
18
TIME OF DAY (h)

Fig. IV.2 Effect of ten injections, 24 h apart, of ACTH (ten ACTH) or saline (ten saline) with the last injection at 7:00 h on day 140 of age on plasma cortisol concentrations from 2:00 h on day 140 of age (Day 1) to 20:00 h on day 141 of age (Day 2) in gilts.

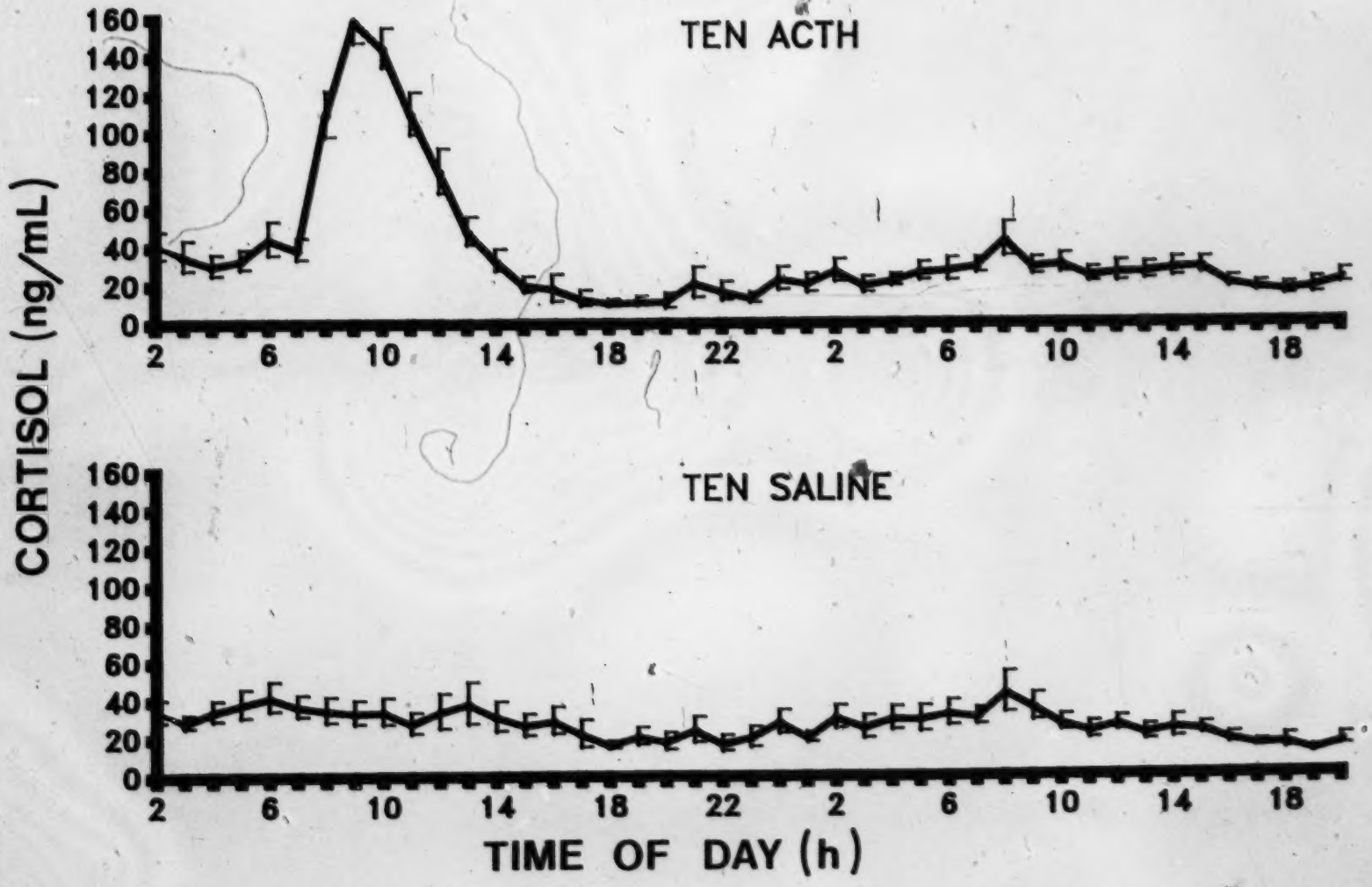

TEN ACTH
TEN SALINE
CORTISOL (ng/mL)
160
140
120
100
80
60
40
20
0
2
6
10
14
18
22
2
6
10
14
18
TIME OF DAY (h)

Fig. IV.3 Example of pulsatile LH profile obtained by blood sampling at 15 min intervals for 4 h from 140 d old gilts.

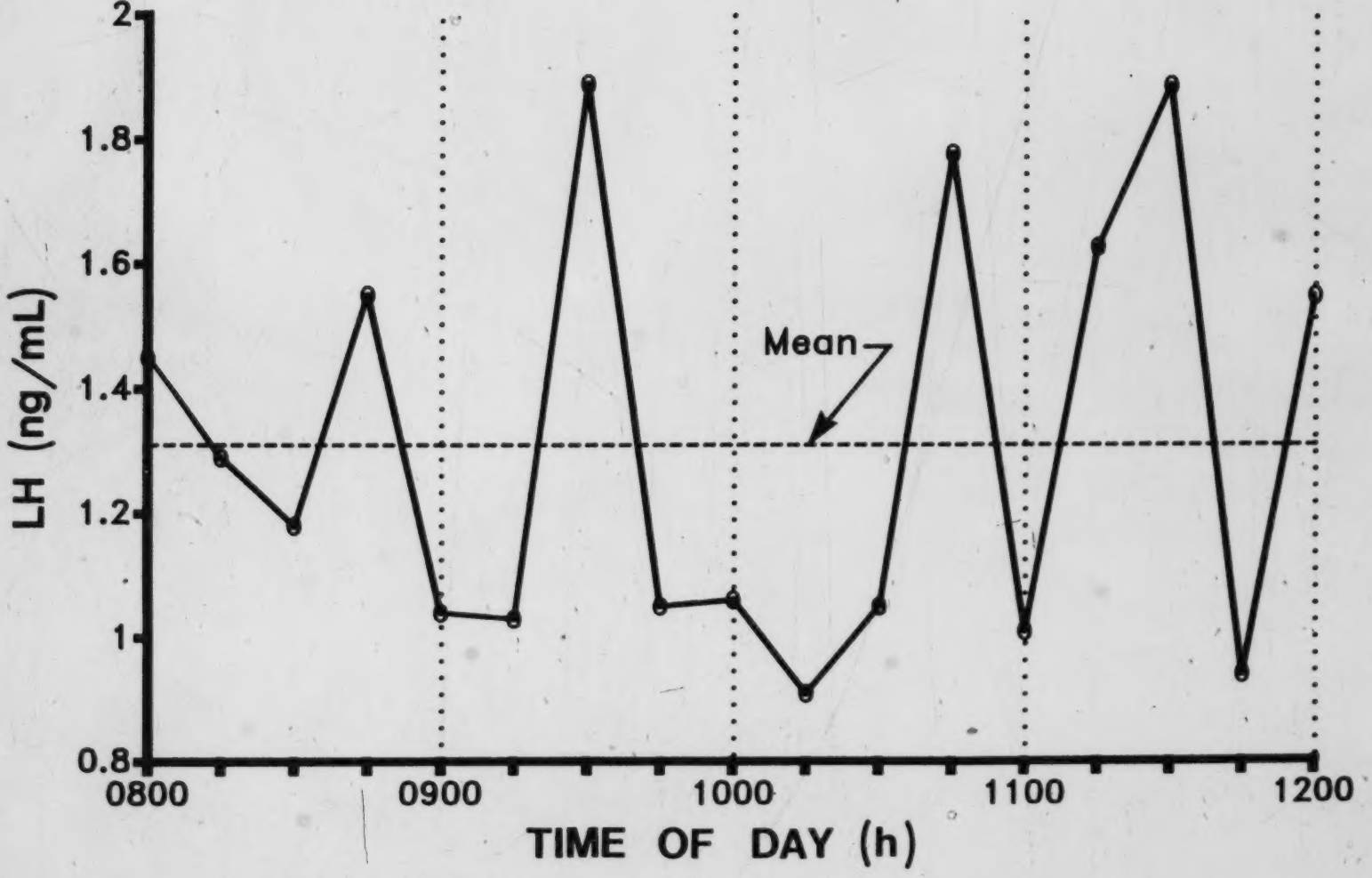
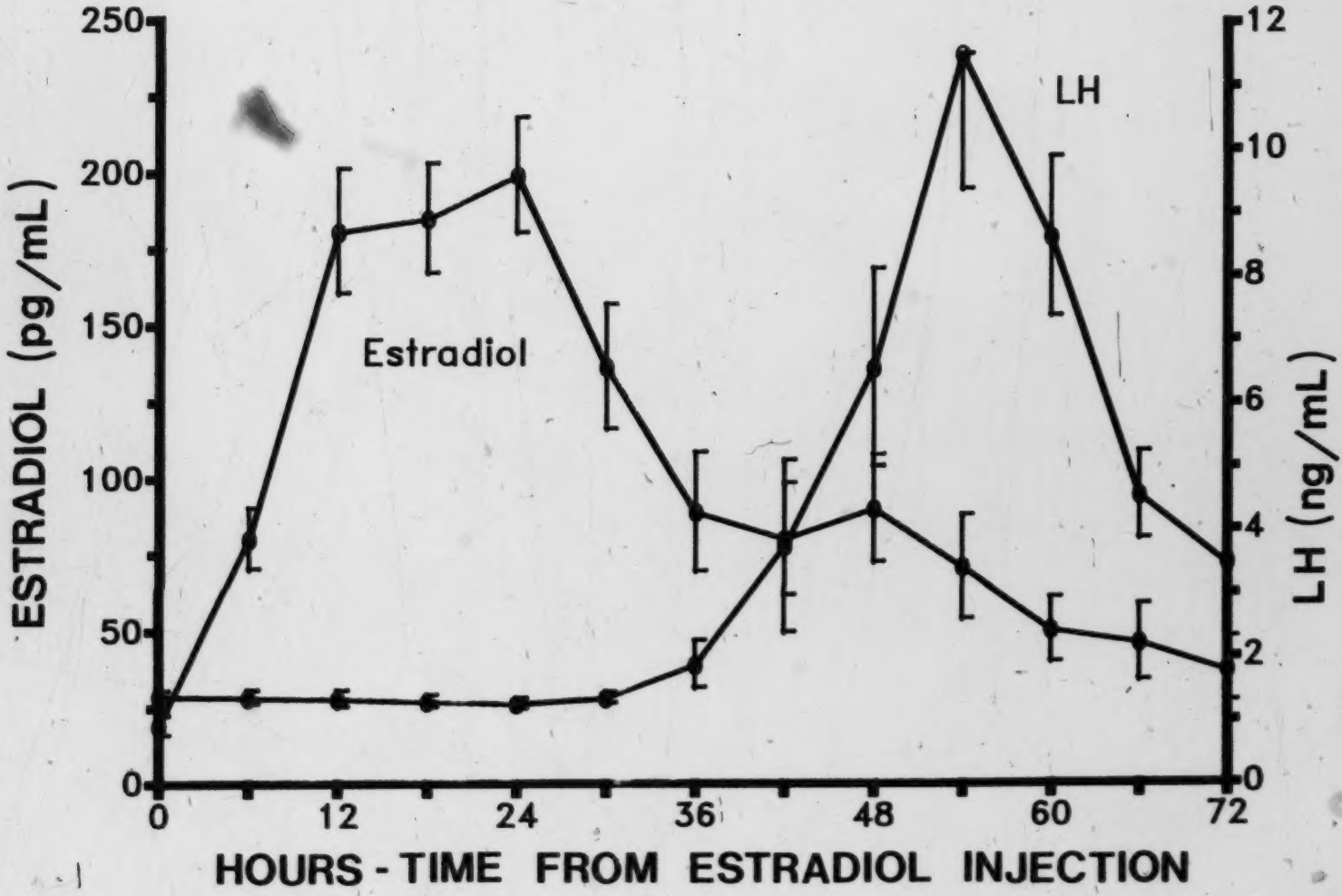

2
1.8
1.6
1.4
1.2
1
0.8
LH (ng/mL)
Mean
0800
0900
1000
1100
1200
TIME OF DAY (h)

Fig. IV.4 Effect of estradiol injection (20 µg/kg live weight 17ß-estradiol-3-benzoate in corn oil) on plasma estradiol-17ß concentrations and the LH surge in 140 d old gilts. Values reflect mean ± SEM pooled across all treatments.


ESTRADIOL (pg/mL)
250
200
150
100
50
0
Estradiol
LH
12
10
8
6
4
2
0
LH (ng/mL)
0
12
24
36
48
60
72
HOURS - TIME FROM ESTRADIOL INJECTION

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V. THE COMPARATIVE PHYSIOLOGICAL DEVELOPMENT OF THE REPRODUCTIVE AND ADRENAL AXES OF THE GILT FROM THREE WEEKS OF AGE TO SEXUAL MATURITY

INTRODUCTION

In the gilt, the reproductive organs including the uterus (Bal and Getty 1970), ovary (Bal et al. 1969) and ovarian follicles (Dyck 1972) undergo a sudden spurt of growth at approximately 12 wk of age. Although much research has been directed toward identifying the agents responsible for initiating prepubertal development, a definitive answer is still lacking (Ojeda et al. 1980).

In rodents, stress, including exposure to light, noise, shaking (Nagy and Arvay 1968), handling (Morton et al. 1963), cold (Mandl and Zuckerman 1952) and restraint (Paris and Ramaley 1974) have been shown to alter the age of vaginal opening. In swine, transport stress and the stress of mixing animals have been reported to induce early puberty in gilts (Bourn et al. 1974; Zimmerman et al. 1974). It is well documented that these as well as other stressors stimulate activity in the adrenal axis (Suzuki 1983). These observations plus others have stimulated interest in determining the role of the adrenal axis in sexual development (Cutler and Loriaux 1980; Ojeda et al. 1980).

Most investigations of the relationship between the

adrenal axis and reproductive axis have utilized the rat or human as the experimental species. Little information is available regarding development of the adrenal axis in swine. The objective of this study was to map the physiological development of the adrenal gland with reference to development of the reproductive axis and other organ systems in the maturing gilt.

MATERIALS AND METHODS

Fifty-five Yorkshire x Landrace gilts were selected at 3 wk of age and randomly assigned to treatment groups. Feed and housing for these animals have been described in Chapter II. One group of ten animals served as control and was raised until sexual maturity without blood sampling. Another group of 10 animals was blood sampled weekly from 3 wk until 30 wk of age. Of the remaining 35 animals, groups of 5 gilts were slaughtered at 4 wk intervals from 4 to 28 wk of age.

Blood samples were collected by suborbital sinus puncture using 18 gauge needles (Monoject 250). After separation at 4°C by centrifugation at 3000 x g for 30 min, the plasma was kept frozen at -20°C until assayed for hormone concentration.

Plasma estradiol-17β was assayed by radioimmunoassay using rabbit antiserum against estradiol-17β (E-26-47, Endocrine Sciences, Tarzana,

CA) as described previously (Chapter IV). Intraassay and interassay coefficients of variation were 6.07% and 13.9% respectively. Porcine LH was assayed by the method described in Chapter IV using rabbit-antibovine-LH. The intraassay coefficient of variation was 5.4%.

All animals in the control and weekly blood sampled groups were monitored daily for signs of estrus from 150 d of age until termination of the experiment. Estrus was confirmed at slaughter by examination of the ovaries.

At the time of slaughter, animals were weighed, then stunned by the use of a captive bolt gun and the carotid artery severed. After opening the abdominal and thoracic cavities the internal organs were removed and separated from connective tissue. The organ weights monitored in this study were the thyroid glands, pineal gland, adrenal glands, heart, lungs, liver, kidneys, spleen and whole reproductive tract including external genitalia. The reproductive tract was further separated into uterus, fallopian tubes and ovaries.

The left adrenal gland was fixed and stored in Bouin's fluid (Drury et al. 1967). A 3 mm cross-section from the centre (sections at right angles to the long axis) of each left adrenal gland was then embedded in paraplast (Fisher Scientific) and 7 µm sections were cut, mounted and stained with haematoxylin and eosin (Drury et al. 1967). Using a microscope and stage micrometer the thickness of each zone of the cortex was determined in six places on

each section. Cell type and zonal characteristics of the adrenal cortex were identified as described by Reith and Ross (1977). To determine changes in cellular synthetic activity, the nucleus to cell volume ratio was determined in each zone of the cortex by the method of Gaunt and Gaunt (1978).

Statistical Analysis

Age at first estrus for control and weekly blood sampled animals were compared by means of Student's t-test.

Average feed efficiency as well as average daily gain for the control and weekly blood sampled animals were compared using two way analysis of variance.

Relative nuclear volume versus age of development for each zone of the adrenal cortex were compared using one way analysis of variance. Thickness of the zona glomerulosa, zona fasciculata and zona reticularis versus age were compared using two way analysis of variance with one repeated measure. Total adrenal cortex thickness was compared according to age of development using one way analysis of variance.

If any analysis of variance indicated a significant F-value, means were compared using Tukey's test. Steel and Torrie (1980) was used as reference for all statistical analysis and differences were considered not significant if $P>0.05$.

RESULTS

Average daily gain for control and blood sampled animals was 0.66 ± 0.02 and 0.68 ± 0.03 kg d^{-1} from 4 to 30 wk of age. Average feed efficiency for the same treatments was 3.50 ± 0.10 and 3.37 ± 0.14 kg feed kg^{-1} gain, respectively. Since neither feed efficiency nor average daily gain were significantly different for control vs blood sampled animals ($P>0.05$), all data for growth parameters were pooled. The combined growth curve for these animals calculated on a weekly basis from 4 to 30 wk of age is shown in Fig. V.1.

Plasma estradiol-17β levels and plasma LH concentrations from 3 to 30 wk of age are shown in Fig. V.2.

Two animals in the control treatment did not reach first estrus by 32 wk of age and age at first estrus was calculated based on the remaining eight animals. Age at first estrus for control gilts (203.4 ± 6.8 d) was not different ($P=0.54$) from that of animals blood sampled (207.8 ± 3.3 d). A scattergram of age vs weight at which first estrus occurred for these animals is also shown in Fig. V.1. When pooled, average age at first estrus was 205.8 ± 3.4 d with an average weight of 125.1 ± 4.2 kg.

Absolute weights of various organs from 4 to 28 wk of age are shown in Fig. V.3. Growth curves for the reproductive tract organs were biphasic, showing growth

spurts at 12 to 16 wk and again at 24 to 28 wk of age while growth curves for the other organs followed the growth curve of the whole animal (Fig. V.1. Relative organ weight, defined as wet weight of organ divided by body weight (Gross 1978) is presented in Fig. V.4 expressed as a percentage increase in relative weight (4 wk of age is reference) with respect to age. The biphasic relationship in relative weight growth curves for the reproductive organs was also present while for the other organs relative weight was constant or decreased.

Relative nuclear volume of the paranchyma cells of the zona glomerulosa, zona fasciculata and zona reticularis of the adrenal cortex are presented in Table V.1. No differences in relative nuclear volume of cells with respect to age in any of the zones could be detected ($P>0.05$).

The data for thickness of the zones of the adrenal as well as total thickness of the adrenal cortex are presented in Table V.2. Thickness of the zona fasciculata, averaged over all ages was significantly greater ($P<0.01$) than either the zona glomerulosa or zona reticularis (7.72 ± 0.34 vs 1.70 ± 0.05 or $2.50 \pm 0.11 \times 10^{-4}$ m).

DISCUSSION

Since age at first estrus did not differ between control and blood sampled animals (203.4 ± 6.8 d vs 207.8

± 3.3 d) it is apparent that the stress of restraint and blood sampling had no effect on attainment of first estrus. From average daily gain and feed efficiency calculations it can also be concluded that the stress involved in weekly blood sample collection was not of sufficient magnitude to affect growth characteristics of these gilts.

There has been much controversy over whether age, liveweight or rate of growth is the best predictor of first estrus in the gilt. It has been suggested that the attainment of puberty is closely connected with the point of inflection on the growth curve (Brody 1945; Monteiro and Falconer 1966). Other researchers however, have suggested that this concept does not appear applicable to the gilt (Goode et al. 1965). Rather than rate of growth, Duncan and Lodge (1960) have suggested that in normally growing gilts, the attainment of puberty is more a function of age than of weight. However, a great variation in age of gilts at first estrus has been reported from as early as 102 d of age (Aherne et al. 1976) to values in excess of 250 d (Warnick et al. 1951). Despite this great range in age at first estrus it has been suggested that chronological age is a significantly better measure of sexual maturity in the gilt than is liveweight (Robertson et al. 1951a; Robertson et al. 1951b).

This strong relationship between age and first estrus in the pig does not however seem to occur in other species

including the human (Frisch and Revelle 1970) or the mouse (Monteiro and Falconer 1966). For these species, body weight is reported to be a better predictor of sexual maturity.

In the present study, when comparing the parameters age vs weight (Fig. V.1) it appears that age is a slightly better predictor of first estrus (205.8 ± 3.4 d, Mean ± SEM) than weight (125.1 ± 4.2 kg, Mean ± SEM) since the coefficient of variation for age (7.0%) is approximately half compared to the coefficient of variation for weight (14.1%). Nevertheless, it is acknowledged that there probably is some critical body weight, even for the gilt, below which sexual maturation does not proceed to completion regardless of the age (Foster and Ryan 1981).

Although in this study first estrus occurred at 29.4 wk of age, from the profiles of LH and estradiol (Fig. V.2) it can be seen that much developmental activity occurred in the reproductive axis prior to final sexual maturation. The profiles of estradiol and LH observed in this study were similar to those reported earlier (Diekman et al. 1983) during pubertal development in the gilt but are more complete since the latter authors commenced sampling at 10 wk of age as compared to 3 wk in the present study.

For the developing female rat, hormone profiles, corresponding to developmental stages of the reproductive axis, have been described previously (Ojeda et al. 1980).

Based on the LH and estradiol profiles obtained in this study and the FSH profile reported by Diekman et al. (1983) the sexual maturation of the gilt can similarly be divided into stages. The first stage, called "infantile", is characterized by the maturation of steroid positive feedback resulting in increasing plasma estrogen, LH and FSH, and appeared to occur from approximately 3 to 14 wk of age. The next stage, called "juvenile", is characterized by increasing sensitivity to the negative feedback of estradiol on gonadotropin release. This stage, characterized by decreasing LH and FSH concentrations leading to reduction in ovarian production of estradiol, appeared to occur from 14 to 20 wk of age. The "prepubertal" stage, characterized by decreasing negative feedback of estradiol on gonadotropin release resulting in increased ovarian development and rising estradiol levels appeared to occur between 20 and 28 wk of age. There was however no indication of an increase in basal levels of LH in the weeks prior to puberty as occurs for estradiol or as suggested by the gonadostat hypothesis. The final stage, "pubertal", involving final maturation of the reproductive axis, full development of ovarian steroid feedback, follicular development and the first LH surge with resulting ovulation, occurred from 28 wk to approximately 32 wk of age in the gilt.

The plasma estradiol and LH profiles (Fig. V.2), showed a transitory increase at 12 wk to 16 wk, dropped

back at 20 wk to 24 wk and increased again at the time of first estrus and were mirrored by changes in reproductive organ weights. From Fig. V.3, it can be observed that very little growth took place in the ovaries, fallopian tubes, uterus or the whole reproductive tract prior to 12 wk of age. Between 12 to 16 wk growth increased, plateaued at 24 wk and thereafter another increase in growth occurred immediately prior to first estrus. The relative growth rate (Fig. V.4) of these same reproductive organs reached a peak at 16 wk of age, decreased along with the decline in gonadotropin levels between 20 and 24 wk (Fig. V.2), and increased again just prior to first estrus. The growth patterns observed in this study were similar to those patterns reported previously for follicular growth in the ovary of the maturing gilt (Dyck 1972).

From Fig. V.3 and Fig. V.4, it can be observed that the growth patterns of the reproductive organs differed markedly from the patterns produced by the other organ systems. Absolute growth curves of the heart, liver, kidney, spleen and lungs appeared to follow closely the whole animal growth curve (Fig. V.1). Growth of these organ systems was reported to be closely related to total metabolism of the organism expressed as a function of body weight or surface area. For example, heart weight was reported to vary as the 0.98th power of body weight while kidney weight varied as the 0.67th power of body weight (i.e. surface area) in a wide variety of species (Price

and White 1985). Relative weight changes of all of these non-reproductive organs were constant or decreased slightly with age (Fig. V.4).

Growth curves for the endocrine glands, the pineal, thyroid and the adrenal followed more closely the growth patterns of other internal organs such as the heart or kidney rather than the reproductive organs (Fig. V.3). Relative weights of these endocrine glands also remained unchanged or decreased with age, unlike the reproductive organs which showed a biphasic increase (Fig. V.4). The growth curve for the adrenal glands in the gilt, therefore, is similar to that reported previously for the human where adrenal weight was found to be highly correlated to body surface area (Dhom 1973).

It is known that in the human, adrenarche, defined as a developmental increase in the adrenal secretion of dehydroepiandrosterone (DHA), DHA sulfate, androstenedione, testosterone and estrone, occurs several years before the onset of menarche (Cutler and Loriaux 1980). Several researchers have suggested that adrenal steroids may play a role in determining the onset of puberty in both the human (Collu and Ducharme 1975) and rat (Gorski and Lawton 1973). More specifically it has been proposed that the increase in adrenal steroids produced during adrenarche is responsible for the decrease in hypothalamic sensitivity to the negative feedback of gonadal steroids leading up to puberty (Ducharme et al.

1976).

If this were true it would seem logical that the adrenal of the gilt at approximately 12 wk to 16 wk of age would initiate or increase synthesis and release of a hormone having an effect on the development of the reproductive axis or on development of the reproductive organs. However, Cutler et al. (1978) have shown that in the pig, as in most other mammals except the human and chimpanzee, DHA, DHA sulfate and androstenedione did not increase during maturation.

In the gilt nuclear to cellular volume ratios, a measure of cellular activity (Nieschlag et al. 1974), did not show any sudden increase in any zone of the adrenal cortex (Table V.1). Although thickness of the zona fasiculata and zona reticularis did change with age (Table V.2), the slight increases were probably only a reflection of overall growth of the adrenal gland rather than preferential growth of one or more zones. It has previously been suggested that thickness of the zones of the adrenal cortex are probably not reliable criteria for activity of the gland (Nieschlag et al. 1974). In Chapter III it was reported that the diurnal rhythm of cortisol did show changes that preceded major reproductive developmental changes. It seems however that the relationship between changes in the adrenal axis and those occurring in the reproductive axis remains equivocal.

It appears therefore, that there is no clear

relationship between growth, morphology or hormonal synthesis and release by the adrenal gland and the periods of rapid growth and development of the reproductive tract during maturation of the gilt. From this study, it would appear that in the gilt, as in the rat or human (Ojeda et al. 1980) there is no compelling evidence for a role of the adrenal cortex in the development of the reproductive axis or the onset of puberty.

Table V.1 Volume of the nucleus of parenchymal cells of the adrenal cortex expressed as a percentage of total cell volume in gilts from 4 to 28 wk of age[1]

Age (wk)	Zona Glomerulosa	Zona Fasciculata	Zona Reticularis
4	41.1 ± 1.6[2,3]	23.1 ± 1.1	29.4 ± 2.9
8	41.0 ± 3.2	21.1 ± 1.8	24.5 ± 2.0
12	39.2 ± 2.0	20.1 ± 1.1	25.2 ± 1.9
16	38.1 ± 2.0	20.3 ± 0.5	22.8 ± 0.9
20	38.9 ± 1.6	19.5 ± 1.2	23.7 ± 1.6
24	41.5 ± 1.6	23.0 ± 2.0	28.1 ± 3.5
28	39.0 ± 2.6	22.7 ± 1.4	26.3 ± 1.7

[1] No significant difference with respect to age of development was detected ($P>0.05$).

[2] Mean ± SEM.

[3] n=5 animals per mean.

Table V.2 Thickness of the adrenal cortex and zones of the adrenal cortex in gilts from 4 to 28 wk of age

Age (wk)	Zones of Adrenal Cortex			
	Glomerulosa	Fasciculata	Reticularis	Total Cortex
4	1.68 ± 0.13a[1,2]	5.55 ± 0.20a	1.92 ± 0.25a	9.16 ± 0.38a
8	1.51 ± 0.11a	6.15 ± 0.56ab	2.62 ± 0.35ab	10.16 ± 0.80ab
12	1.79 ± 0.19a	7.31 ± 0.38cd	2.39 ± 0.14ab	11.50 ± 0.33abcd
16	1.59 ± 0.09a	8.63 ± 0.44de	2.91 ± 0.13ab	13.14 ± 0.55bcd
20	1.84 ± 0.10a	8.84 ± 0.73e	3.06 ± 0.41b	13.75 ± 1.05bc
24	1.84 ± 0.11a	7.10 ± 0.36bc	2.11 ± 0.08ab	11.06 ± 0.41acd
28	1.65 ± 0.10a	10.42 ± 1.03f	2.55 ± 0.20ab	14.63 ± 1.11b

a-f Means in the same column without a common letter differ ($P<0.05$).

[1] Units of thickness 10^{-4} m.

[2] n=5 animals and 6 observations per animal per mean (± SEM)

Fig. V.1 Growth curve calculated on a weekly basis and age/weight relationship for control and weekly blood-sampled gilts from 4 to 32 wk of age (n=20). The asterisks denote the age and weight at which first estrus occured in the 18 animals that attained puberty.

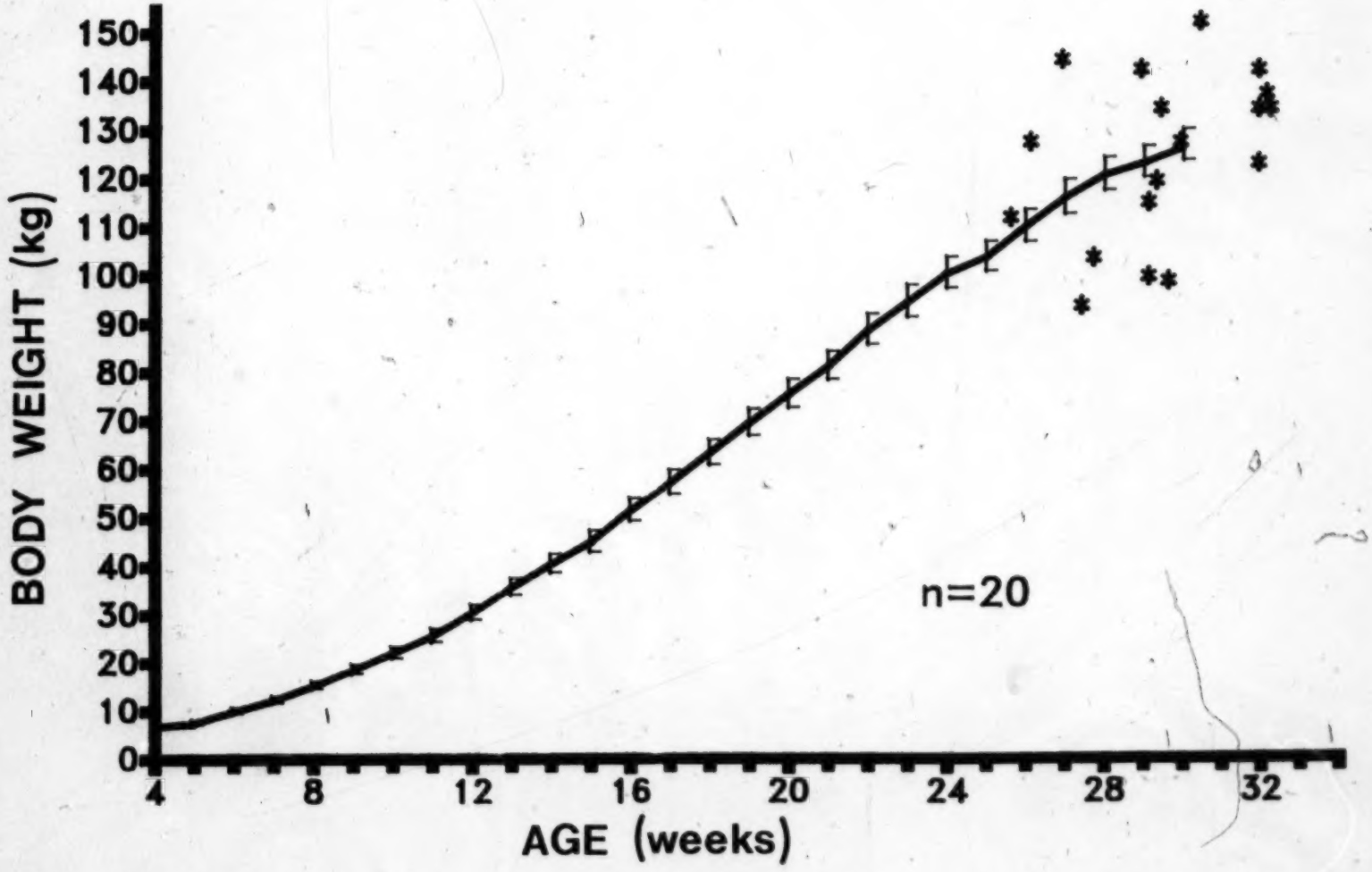
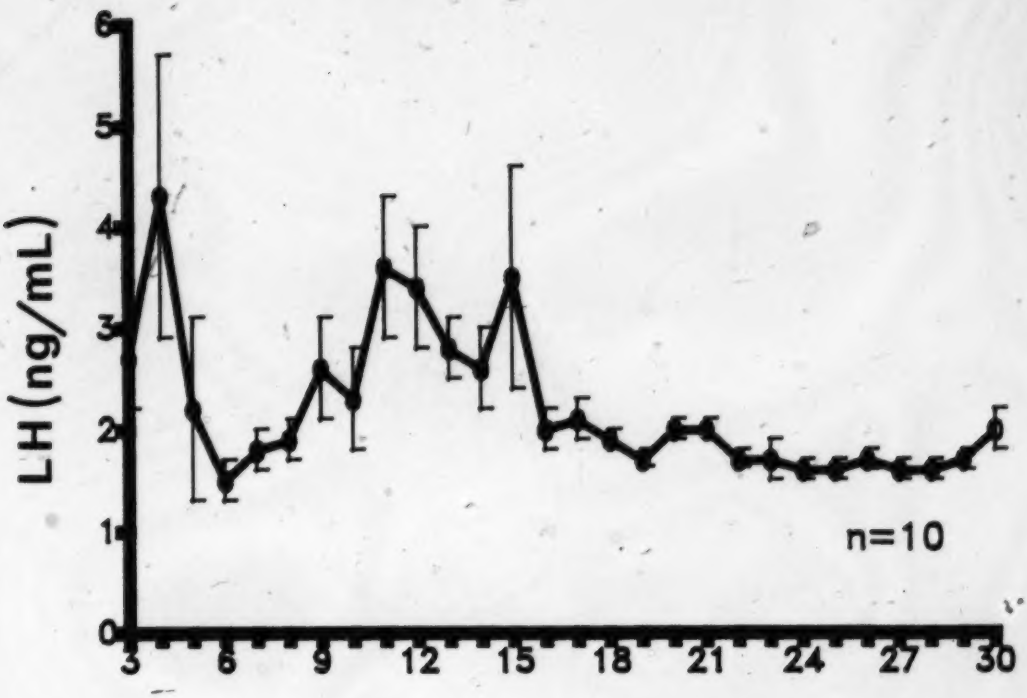

BODY WEIGHT (kg)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
4
8
12
16
20
24
28
32
AGE (weeks)
n=20

Fig. V.2 Plasma profiles of estradiol-17β and LH from 3 to 30 wk of age in the gilt.


LH (ng/mL)
0
1
2
3
4
5
6
n=10
3
6
9
12
15
18
21
24
27
30

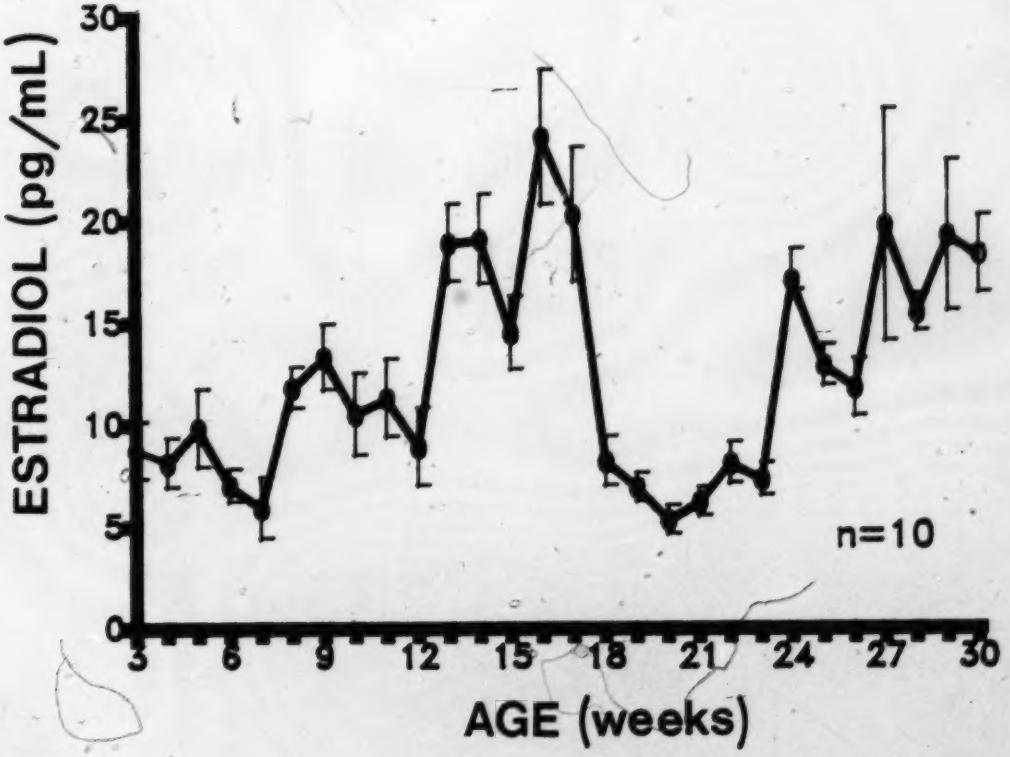

ESTRADIOL (pg/mL)
0
5
10
15
20
25
30
n=10
3
6
9
12
15
18
21
24
27
30
AGE (weeks)

Fig. V.3 Absolute organ weights (mean + SEM) in gilts slaughtered in intervals from 4 to 28 wk of age.

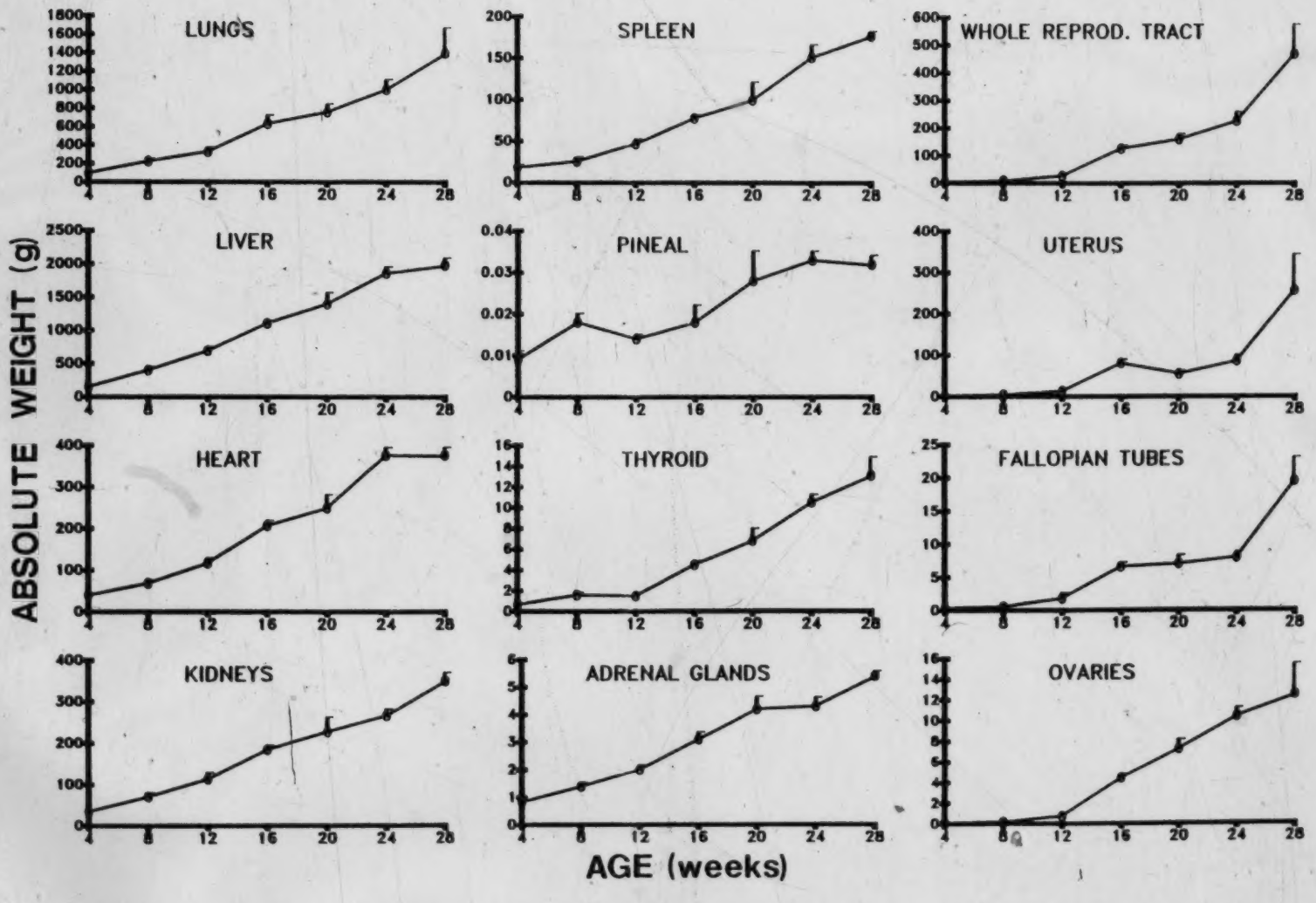
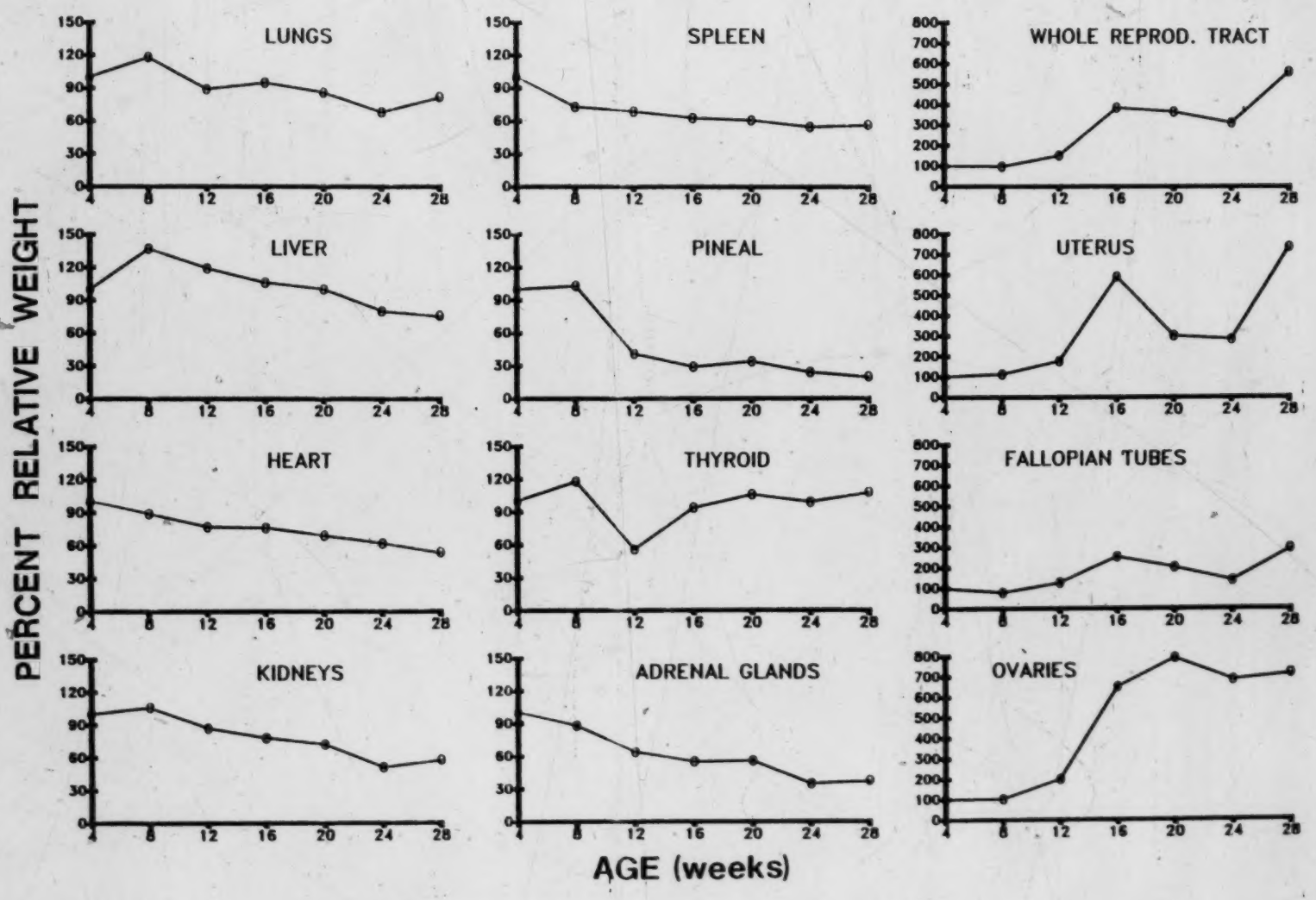

LUNGS
SPLEEN
WHOLE REPROD. TRACT
LIVER
PINEAL
UTERUS
HEART
THYROID
FALLOPIAN TUBES
KIDNEYS
ADRENAL GLANDS
OVARIES
ABSOLUTE WEIGHT (g)
AGE (weeks)

Fig. V.4 Relative organ weights (wet weight divided by body weight), expressed as a percentage of weight at 4 wk of age in gilts from 4 to 28 wk.


PERCENT RELATIVE WEIGHT
LUNGS
SPLEEN
WHOLE REPROD. TRACT
LIVER
PINEAL
UTERUS
HEART
THYROID
FALLOPIAN TUBES
KIDNEYS
ADRENAL GLANDS
OVARIES
AGE (weeks)

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VI. GENERAL DISCUSSION

Most of what we know about sexual maturation and the processes involved in reproduction has been obtained from experiments using the laboratory rat as the experimental species (Ojeda et al. 1980). The results of these studies have provided the basic information to formulate hypotheses explaining, for example, what factors govern maturation of the hypothalamo/pituitary/gonadal axis. These theories often form the basis of explanations for solving pathological problems in human development or in formulating more elaborate theories to explain the function of the reproductive axis in all species. However these theories should be tested for validity in species other than those in which they originated.

In Chapter II it was observed that 17ß-estradiol receptor (E_2R) concentrations in the cytosolic fraction from tissue samples of the hypothalamus and pituitary do not increase during maturation of the gilt. This observation that in the gilt E_2R concentrations in the hypothalamus are low and do not increase from weaning to sexual maturity are supported by Diekman and Anderson (1982). These results are clearly contradictory to the observation that an increase in cytosolic E_2R concentration occurs with age in the anterior and central regions of the rat hypothalamus (Kato et al. 1974). This observation in the rat has formed the basis for a general

hypothesis explaining the mechanism of sexual maturation. The hypothesis suggests that the increases in E_2R concentration in particular regions of the hypothalamus are responsible for changes in the setpoints of the positive and negative feedback effect of estradiol on gonadotropin release (Kato 1980). This change in setpoints of feedback due to changes in levels of receptors is said to explain the onset of puberty in the human and other species by the gonadostat theory of sexual maturation (Ojeda et al. 1980). From the results of Diekman and Anderson (1982) and from those of Chapter II it can be concluded that for swine the E_2R theory of puberty onset is not applicable. This casts serious doubt on the validity of extrapolating the receptor theory of pubertal development from the rat to the human or any other species. Perhaps the rat and mouse are the only species in which E_2R concentrations increase during sexual development and perhaps these increases are not connected with changes in feedback sensitivity responsible for gonadotropin release.

It is also conceivable that other possibilities exist to explain this discrepancy in receptor profiles. In the rat, E_2R sites in the hypothalamus and brain have been extensively mapped (McEwen 1976). Perhaps in swine areas of the brain other than the hypothalamus may be involved with centres of positive and negative feedback. To date very little mapping of specific E_2R sites has been done

in any species other then the rat and none has been reported for swine. One efficient way to map receptor sites using currently available techniques might be to employ monoclonal antibodies specific to active receptors. Application of these specific antibodies, subsequently labelled with fluorescent molecules coupled to second antibodies and validated by blocking techniques, might be a more specific and efficient method than using autoradiographic techniques employed in the past.

What must also be examined is the principle that E_2R concentration is related to the response of a tissue to a particular hormone (Mulvihill and Palmiter 1977). Perhaps the limiting factor is the processing of the information within the cell or expression of cellular activity in response to the presence of the hormone in the cell's environment. It is also possible that estradiol-17β is not the hormone involved in the feed back response within cells of the central nervous system. McDonald and Doughty (1974) have suggested that uptake of steroid as androgen, followed by intracellular conversion by aromatization to estrogen and binding to classical estrogen receptors, might be involved in the central actions of steroids on reproductive function. Kato (1980) has also suggested that, because of the presence of other receptors in the brain, for example, 5-alpha-dihydrotestosterone receptors, that it is neither testosterone nor estradiol-17β that is sought by a classical receptor system but a metabolite

of those steroids.

In contrast to E_2R levels the development of the circadian rhythm of glucocorticoid release during maturation in swine (Chapter III) is similar to the pattern observed in rats (Takahashi et al. 1979). It is interesting that in Chapter III full development of the hormonal output of the adrenal axis of the gilt did not occur until the time of or shortly before first estrus. This evidence, although circumstantial, would tend to support the suggestion that the development of the hypothalamo/pituitary/adrenal axis is associated with development of the hypothalamo/pituitary/gonadal or reproductive axis.

However, investigation of the effects of activation of the adrenal axis by ACTH on release of LH from the reproductive axis has not supported the hypothesis that stress, acting through the adrenal axis, can effect gonadotropin release (Chapter IV). This has been the theme common to both proponents of the stress theory of puberty induction (Thibault et al. 1966; Signoret 1972; Bourn et al. 1974; Zimmerman et al. 1974) and to those suggesting that stress has a negative effect on reproduction (Liptrap 1970; Barb et al. 1982; Li and Wagner 1983). It can be concluded from Chapter IV that, in the young prepubertal gilt, ACTH activation of the adrenal axis had no effect on the plasma concentrations or release patterns of the gonadotropin LH which has been implicated in the timing of

puberty in other species (Ryan and Forster 1980; Chipman 1980).

It was also shown (Chapter IV) that ACTH administration had no effect on a major feedback loop in the reproductive axis. This loop, the positive feedback effect of estradiol on the LH surge, is responsible for ovulation (Ramirez 1973). It remains to be investigated whether ACTH stimulation might have effects on the release of FSH, the gonadotropin responsible for follicular development. This is an important question, especially in veiw of the recent evidence suggesting that glucocorticoids have differential effects on FSH and on LH release (Suter and Schwartz 1985).

In addition to the lack of effect ACTH administration had on LH release from the reproductive axis (Chapter IV), no evidence could be provided for participation of the adrenal axis in the timing of puberty (Chapter V). There was no evidence to suggest that any parameter associated with the adrenal cortex including growth of the gland, thickness of the zones or activity of the cells in the zones, increases in a manner to account for production of a hormone or factor responsible for reproductive axis development.

From the collective results of these studies it can be concluded that there is no evidence to suggest that any component of the hypothalamo/pituitary/adrenal axis below ACTH release has an effect, either positive or negative,

on reproductive axis function. This does not mean however, that stressors do not have an effect on reproductive axis maturation, but that their effect might be exerted at a level of the axis higher than pituitary release of ACTH. In fact, stress may act to stimulate activity of neurons located in portions of the central nervous system common to the control of several neuroendocrine axes.

In Chapter IV it was reported that ACTH administration had no effect on subsequent basal levels of plasma cortisol in the gilt. It was also suggested that control centers in the adrenal axis above ACTH release might be more influential in altering hormonal release during actual periods of stress. Under actual stress conditions it has been observed that for some stressors, for example, endotoxin administration in dogs (Egdahl 1964) or anoxia in rats (Dallman and Yates 1968) that pituitary-adrenal response could not be suppressed by pretreatment with corticosteroid administration. The response to other stressors, however, such as severe hemorrhage (Gann and Cryer 1973) or administration of histamine (Dallman and Yates 1968) could be suppressed by pretreatment with corticosteroids. It has been interpreted that those stimuli that were steroid sensitive were transmitted within the neural component of the adrenal axis via different neural pathways as compared to the non-steroid sensitive stressors (Keller-Wood and Dallman 1984). If these multiple neural pathways are proven to exist it

would suggest that the route of signal transmission from transduction of an external stimulus to the release of releasing factors from the hypothalamus may be much more complex than originally thought.

The existance of multiple neural pathways could explain why, under specific conditions, a stressor or combination of stressors, for example, during transport or mixing of gilts (Bourn et al. 1974; Zimmerman et al. 1974), could excite not only the adrenal axis but also the reproductive axis leading to early onset of puberty. In addition, this model could explain why activation of the adrenal axis at a level below neural involvement, such as by ACTH administration, would have no effects on the reproductive axis (Chapter IV) or under pharmacological circumstances, it could have negative effects on reproduction (Barb et al. 1982)

Multiple neural pathways are not the only complicating factors to consider in trying to understand function of the adrenal axis. It now appears that ACTH is not the only product of CRF stimulation of the pituitary. Roberts et al. (1982) reported that the region on the chromosome that codes for ACTH is part of a gene that codes for a much larger protein called proopiomelanocortin (POMC). POMC in turn is processed by proteolytic cleavage to ACTH, ß-endorphin, ß-lipotropin and other peptides that vary with the cells in which processing is taking place. It has previously been shown that ß-endorphin

and ACTH are secreted concomitantly by the pituitary under a wide variety of circumstances (Guillemin et al. 1977). The effects of ß-endorphins as well as other opiate peptides have been reported to include, among other effects, stimulation of gonadotropin release by inhibiting the effects of dopamine, an inhibiting neurotransmitter for GnRH release (Hutchinson 1980). In addition to the effects of these peptides released with ACTH, there is mounting evidence for the suggestion that CRF is not mearly a releasing factor for ACTH release but is, itself, a neurotransmitter (Rivier and Plotsky 1986). These observations indicate that interactions between neuroendocrine axes may occur not only through common and interacting neurons but also by activation from peptides or neurotransmitters released concomitantly during activation of another neuroendocrine axis.

Future research into the effects of stress on puberty induction should therefore be directed towards identifying those neural pathways implicated in transducing specific environmental stimuli into endocrine signals. A study of these pathways and neurotransmitters involved may provide for the discovery of common or interconnecting neurons and common neurotransmitters or compounds which could lead to activation of axes other than the primary one. This research would lead to a more integrated study of the interaction and interdependence of the neuroendocrine axes rather than studying each axis in isolation as has been

frequently done in past research. It would seem that these investigations will require intensive experimentation, well equipped laboratories, sophisticated techniques and advanced instrumentation. The benefits however, of being able to control and manipulate the neural and endocrine milieu of an organism in a predictable manner would seem to be enormous.

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APPENDIX

Table 1. Composition and analysis of the starter pig diet fed from weaning to 20 kg live weight

Ingredients	Percent
Wheat	20.0
Barley	56.3
Soybean meal	19.3
Tallow	0.4
Dicalcium phosphate	1.5
Calcium carbonate	1.0
Salt (iodized)	0.4
Vitamins and minerals[1]	1.0
L-Lysine	0.05
Chemical Analysis[2]	**Percent**
Dry matter	88.6
Crude protein	18.1
Crude fiber	4.4
Lysine	0.98
Digestible Energy[3] (MJ DE kg^{-1})	12.7

[1] Supplied per kg of diet: Vitamin A, 5000 IU; Vitamin D_3, 500 IU; Vitamin E, 22 IU; riboflavin, 12 mg; niacin, 45 mg; biotin, 200 µg; calcium pantothenate, 25 mg; Vitamin B_{12}, 30 µg; ASP250, 275 mg; zinc, 120 mg; manganese, 12 mg; iron, 150 mg; copper, 12 mg; selenium, 0.1 mg; choline chloride, 500 mg.

[2] Determined values reported on as-fed basis.

[3] Calculated from gross energy determination and reported on as-fed basis.

Table 2. Composition and analysis of the growing pig diet fed from 20 to 50 kg live weight

Ingredients	Percent
Wheat	40.0
Barley	43.3
Soybean meal	13.2
Dicalcium phosphate	1.2
Calcium carbonate	1.2
Salt (iodized)	0.5
Vitamins and minerals[1]	1.0
Chemical Analysis[2]	**Percent**
Dry matter	87.8
Crude protein	16.1
Crude fiber	4.3
Lysine	0.73
Digestible Energy[3] (MJ DE kg^{-1})	13.2

[1] Supplied per kg of diet: Vitamin A, 5000 IU; Vitamin D_3, 500 IU; Vitamin E, 22 IU; riboflavin, 12 mg; niacin, 45 mg; biotin, 200 µg; calcium pantothenate, 25 mg; Vitamin B_{12}, 30 µg; ASP250, 275 mg; manganese, 120 mg; iron, 150 mg; copper, 12 mg; choline chloride, 500 mg.

[2] Determined values reported on as-fed basis.

[3] Calculated from gross energy determination and reported on as-fed basis.

Table 3. Composition and analysis of the finishing pig diet fed from 50 to sexual maturity

Ingredients	Percent
Wheat	40.0
Barley	48.6
Soybean meal	7.0
Stabilized fat	0.5
Dicalcium phosphate	1.2
Calcium carbonate	1.2
Salt (iodized)	0.5
Vitamins and minerals[1]	1.0
Chemical Analysis[2]	**Percent**
Dry matter	85.7
Crude protein	14.2
Crude fiber	4.3
Lysine	0.67
Digestible Energy[3] (MJ DE kg^{-1})	13.3

[1] Supplied per kg of diet: Vitamin A, 5000 IU; Vitamin D_3, 500 IU; Vitamin E, 22 IU; riboflavin, 12 mg; niacin, 45 mg; biotin, 200 µg; calcium pantothenate, 25 mg; Vitamin B_{12}, 30 µg; manganese, 120 mg; iron, 150 mg; copper, 12 mg; choline chloride, 500 mg.

[2] Determined values reported on as-fed basis.

[3] Calculated from gross energy determination and reported on as-fed basis.